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Arundo donax water use efficiency and photosynthetic response to drought

Thesis submitted for the Degree of
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The author

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Eficiência no uso de água e resposta fotossintética à seca no *Arundo donax*

A autora

Andreia Garreiro

À Avó Maria

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To whom it may concern

Dr. Walter Zegada-Lizarazu supervised the work of Andreia Garreiro in the University of Bologna along with Prof. Andrea Monti of the same institution. According to the rules of Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa (FCT-UNL), only two supervisors can be shown in the front page of the document, and one of them must be a Professor from FCT-UNL, linking the Faculty with the student. Because of this rule, the name of Dr. Walter Zegada-Lizarazu cannot appear in the front page, but I can hereby confirm that Dr. Walter Zegada-Lizarazu did had an important role as co-supervisor of Andreia Garreiro while she attended the University of Bologna under an ERASMUS agreement between FCT-UNL and University of Bologna.

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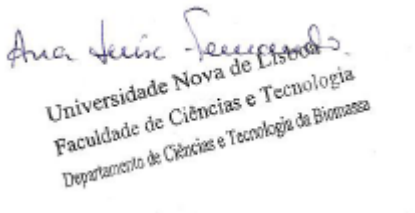

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RESUMO

Arundo donax L., comumente conhecido como Cana-do-reino, tem sido identificado como uma excelente matéria-prima para a obtenção de biomassa, devido aos seus altos rendimentos e baixo requisito de nutrientes. Têm sido apontadas como desvantagens principais para o uso desta matéria-prima na obtenção de bionergia a elevada necessidade de recursos hídricos de maneira a sustentar um rápido crescimento e o seu caractér invasor. No entanto, existem poucos estudos para quantificar as suas necessidades hídricas. O objectivo deste estudo passa pela melhor compreensão da capacidade de obtenção de água pela raízes mais profundas e da eficiência do uso de água pelo Arundo donax. Ainda foi possível avaliar os efeitos de seca na eficiência fotossintética do Arundo donax. Foi imposto um período de seca às camadas superiores da raíz ao longo do tempo e comparado com plantas normalmente irrigadas (irrigação total da raíz). Foi possível avaliar a evolução da experiência através do recurso a doze *rhizotrons* cilíndricos separados em dois compartimentos independentes por uma camada de *Vaseline*. A humidade do solo foi ajustada de maneira a impôr seca à camada superior do solo (topsoil) enquando a camada mais profunda manteve-se normalmente irrigada. A eficiência relativa à captação de água aumentou e a eficiência fotossintética manteve-se, nas plantas sujeitas a seca. A eficiencia da captação de água pelas raízes mais profundas aumentou também. No entanto, não se observou um aumento da densidade das raízes. Em conclusão, podemos afirmar que a resistência à seca do Arundo donax e a manutenção da máquina fotossintética deve-se em parte ao aumento tanto da eficiência de captação da água de toda a planta como ao aumento da eficiência de captação das raízes mais profundas e não devido ao aumento da densidade das raízes mais profundas.

ABSTRACT

Arundo donax L., commonly known as giant reed or Giant reed, has been identified as an excellent biomass feedstock, because of its high yields delivered from low nutrient inputs. Two criticisms of cultivating *Arundo donax* are that it has a history of biological invasion, and also that it may require great quantities of water to sustain its rapid growth. Yet, there is little research reported quantifying the water-use requirements. The objective of this study was to better understand the deep water uptake ability and water use efficiency (WUE) of *Arundo donax*. Moreover, it was possible to evaluate the effects of drought on the photosynthetic efficiency of *Arundo donax*. Durable transient drought stress period were imposed a long time to top soil layers and compared with well watered plants. Twelve cylindrical transparent rhizotrons, separated in two parts by a Vaseline layer allowed to follow the evolution of this experiment. Soil moisture was adjusted to well-watered and drought conditions in the upper (topsoil) layer, while the lower (deep) layer was always wet. WUE was significantly increased in plants of *Arundo donax* by drought and the photosynthetic efficiency was not affect by drought. Relative water uptake from deep soil layers and water use efficiency of deep roots were significantly increased by drought in top soil layers. However, the increase of RLD in deep roots was not observed. In conclusion, the drought resistance of *Arundo donax* and the maintenance of the photosynthetic apparatus are explained by higher WUE and higher WUE of deep soil layers but not because deep soil roots enhanced root length density.

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1. INTRODUCTION

In recent years, many national and international initiatives have started in order to identify new sources of renewable energy. This growing interest in renewable energy is driven by two main reasons. Firstly, fossil fuels such as oil, coal, and natural gas are limited resources on your planet and if the level of our consumption does not change, the estimated times of depletion of these energy sources will be approximately 50 years for oil, 70 years for natural gas and 170 years for coal (International Energy Outlook, 2006). Secondly, the combustion of fossil fuels emits large amounts of gas into the atmosphere, increasing the natural greenhouse effect. Carbon dioxide (CO_2) and methane (CH_4) are the main components of greenhouse gases (GHGs). Since 1870, annual CO_2 emission from fuel combustion dramatically increased from near zero to 29 Gt in 2007 (International Energy Agency, 2009). In this context, the use of biomass for fuel is proposed as one of the options to reduce greenhouse gas (GHG) emissions. The European Commission encourages the use of biomass: the last directive from the EC states that the Member State of the EU should gradually increase the consumption of biomass and by 2020, 20% of EU energy consumption should be from renewable resources (Directive 2009/28/EC). In particular, in the European agricultural sector, the spread of non-food crops for energy production could provide a good opportunity for the integration of income. In Europe, the cropping of high yield biomass plants (energy crops) on less rich land and fallows can provide farmers with a subsidized opportunity to diversify their production, increase their profit margins and hedge their financial risks without compromising the food supply margins (McKendry *et al.,* 2002). These plants can potentially be grown in large fields and reduce carbon emissions from fossil fuels. Perennial, rhizomatous grasses display several positive attributes as energy crops because of their high productivity, low demand for nutrient inputs due to the recycling of nutrients by their rhizomes, and their tolerance to biotic and abiotic stress (Renz *et al.*, 2009). Among these grasses, giant reed (*Arundo donax* L.) is of special interest as an energy crops because it ranked first in some comparative studies for yield (Lewandowski *et al*., 2003). Giant reed is a strong candidate to be used as a renewable biofuel source because of its fast growth rate, ability to grow in different soil types and adaptability to different climatic conditions (Angelini *et al.*, 2005).

1.1 WATER USE EFFICIENCY

With the change of global environment, the impact of drought stress on crop yield becomes more significant. Thus, for sustainable agricultural development, irrigation practices must be established on the basis to use the available water resource more efficiently. During the last two decades, a great deal of work has been done to try to improve plant water use efficiency (WUE) (Chaves *et al.,* 2002). WUE can be observed at several levels. At a single leaf level, viz. WUE_{leaf} can be defined as the ratio between the rate of light-saturated photosynthesis (Amax) and the rate of stomatal conductance (g_s) for water vapour. Theoretically, WUE_{leaf} may be improved by partial closure of stomata so that intercellular CO_2 concentration (C_i) is just sufficient for saturation of Amax while the rate of water loss had been significantly lowered (Farooq et al., 2009). At whole plant level, viz. WUE_{plant} is determined as the ratio between dry mass (DM) accumulation and plant water use, i.e. transpiration (E).

It is well known that plants can sense the water availability around the roots and respond by sending chemical signals to the shoot to elicit several adaptive responses, including decrease in leaf expansion growth and stomatal closure, and this can occur without any change in shoot water relations could be detected (Hartung *et al.,* 2003). Accumulated evidence suggests that drought-induced increase in $[ABA]_{xylem}$ acts as a major signaling molecule involved in the response of plants to drought stress (Zhang *et al.,* 1991). A common functional mode of the ABA signaling in drought-stressed plants has been proposed: as the soil dries, ABA is produced in the root tips and is transported into the xylem via the transpiration stream reaching the leaf where it reduces stomatal conductance. As consequences of these, the aperture of stomata can be regulated so that a partial closure of stomata at a certain level of soil water deficit may lead to an increase in WUE (Liu *et al.,* 2003).

1.2 DROUGHT TOLERANCE IN CROP PLANTS

One of the most important constraints for agriculture is water limitation. More recently, global warming may be worsening this situation in most agricultural regions. Thus, it is quite relevant to understand the mechanisms that enable plants to cope with water deficit. Indeed, plants show a wide range of adaptations, at different levels, to drought stress.

Drought stress has a tremendous effect on agriculture, thus negatively influencing human activities (NOAA, 2003). During crop domestication, it is assumed that plants were selected on the basis of different traits, water limitation tolerance being unlikely one of them. Historical evidence links drought with the decay of Mayan civilization from 550 to 570 A.C. (Haug *et al.*, 2003). More recently, drought episodes have resulted in extensive fires in North and Central America as well

as in other regions of the world; a drought in northern China in 1878 dried up crops in an extensive region.

Since it is predicted that the world precipitation pattern will change, either heavy rain or drought are similarly expected in long term. Therefore, drought will continue being a problem to be solved for crop production, since water limitation causes stress in plants and limits the production of important cultivations worldwide (Hoerling *et al.,* 2003). The need for new alternatives for a sustainable agriculture, such as drought-tolerant plants, will provide a practical solution to alleviate the problem of water limitation. Most of the alternatives are based either on accelerating the selection of natural varieties or/and inserting genes from other plant varieties or species with the capacity to provide drought tolerance. To achieve this goal, the biological base for drought tolerance needs to be understood (Lambers *et al.*, 2000).

Factors controlling stress conditions, like drought, alter the normal equilibrium, and lead to a series of morphological, physiological, biochemical and molecular changes in plants, which adversely affect their growth and productivity. The average yields from the major crop plants may reduce by more than 50% owing to stresses (Rodriguez *et al.,* 2004) However, plants also have developed innate adaptations to stress conditions with an array of biochemical and physiological interventions that involves the function of many stress-associated genes.

Water, comprising 80-90% of the biomass of non-woody plants, is the central molecule in all physiological processes of plants by being the major medium for transporting metabolites and nutrients. Drought is a situation that lowers plant water potential and turgor to the extent that plants face difficulties in executing normal physiological functions. However, a few groups of animals and a wide variety of plants are known for their tolerance to desiccation during the adult stages of their life cycle (Chaves *et al.,* 2004).

1.2.1 Water Uptake and Movement through Plants

Water is constantly moving from the soil, into plant roots, and through the xylem tissues of the stem to leaves where it is ultimately lost to the atmosphere during transpiration. This cycle is referred to as the soil–plant–atmosphere continuum (SPAC). When water moves through the SPAC, it travels through different mediums (including cell wall, cell membrane and air spaces) at different distances, which utilize different modes of transport. There are three principal modes of water transport: diffusion, mass flow and osmosis. In diffusion, water molecules move spontaneously from regions of high concentration to regions of lower concentration, i.e. along a concentration gradient. This movement is rapid over a short distance and thus drives short-distance transport, for example between cells and during the loss of water to the atmosphere from leaf stomata. In mass flow, groups of water molecules move under an external force, such as a build-up of pressure that forms a gradient. Mass flow

of water is the predominant mode by which long-distance transport of water in stems is accomplished. It also accounts for much of the water flow though the soil and through the cell walls of plants. The third mode, called osmosis, is movement of water molecules through a semipermeable membrane, an example of which is the cytoplasmic membrane. Osmosis occurs spontaneously in both short- and long-distance transport as a response to driving forces of concentration (as in diffusion) and pressure gradient (as in mass flow) (Farooq *et al.,* 2009).

These driving forces of water movement of both osmotic (concentration) and mass flow (pressure) origin are collectively known as water potential. Water potential is measured in units of pressure or suction, i.e. force per unit area required to move a specific amount of water. The most common unit used for studying soil water potentials in the field is kilopascals (kPa). The movement of water in the SPAC is thus dependent on differences in water potential between surrounding soil and plant or atmosphere. Often, the water potential gradient is directed from the roots towards the shoot, as transpiring leaves exposed to the atmosphere have the lowest water potential. However, under situations when the soil is too dry this water potential gradient could be reversed, resulting in loss of water from plant roots to the soil. Also any environmental factors that influence the transpiration of the leaf stomata, e.g. wind or increase in temperature may further decrease the leaf water potential further, speeding up water loss (Yordanov *et al.*, 2003).

1.2.2 Water Dynamics of the Plant Cell

The water within the cell is defined in terms of its free energy content or ability to do work. The free energy per unit volume of water is the water potential (Ψ_w). Water is taken into the plant if the water potential is less than that of the environment surrounding the cell (Figure 1), since water moves down a chemical gradient (Parry *et al.,* 2002). If the water potential in the soil solution is higher than that of the cells, water can be transported into the cells of the root. The water potential of the cell is dependent upon two important parameters: the osmotic potential (Ψ_s) and the turgor pressure (Ψ_p). The content of solutes in the water of the cell (Ψ_s) and the pressure of the cellular contents against the cell wall (Ψ_p) decrease the water potential. An additional component, the matric potential (Ψ_m), or the binding of water to surfaces, also reduces the cell water potential. Equation (1) is used to describe cell water potential.

$$\psi_w = \psi_s + \psi_p + \psi_m \qquad (1)$$

Changes in cellular water relations trigger further events that are manifested in plant responses at the molecular, metabolic, cellular, physiological and developmental levels.

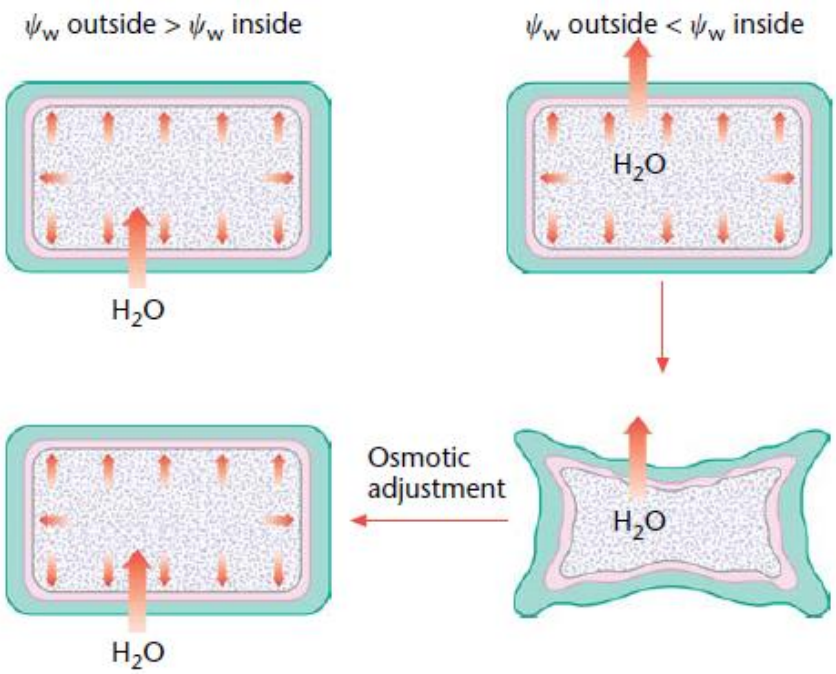

Figure 1 – Cellular and soil-water potential control water uptake into the cell. Osmotic adjustment, a lowering of cellular osmotic potential, can permit water uptake and restore cellular turgor (Parry *et al.,* 2002).

In a process called osmotic adjustment, metabolism may be altered to maintain cellular water content through an increase in the concentration of solutes. In these cells, the osmotic potential is lowered and thus the water potential of the cell is lowered, permitting water uptake to be maintained (Figure 1). Turgor will fully or partially recover depending upon the external water potential. The cells will avoid a loss of water, yet they must be able to withstand low cellular water potential. The solutes that accumulate, called osmolytes, include sugars, proline and quaternary ammonium compounds such as glycine betaine (Parry *et al.,* 2002). They are generally thought to be neutral to metabolic processes, and therefore do not disrupt plant function. The ability to adjust osmotically is dependent upon the genotype and is a more successful defence in resistant genotypes. Interestingly, osmolytes may have additional functions in stress resistance including the ability to stabilize proteins and ameliorate oxidative stress that may arise when

plants are subjected to water deficit. Plants engineered to produce more glycine betaine have improved photosynthetic capacity when grown under water-deficit conditions (Passioura *et al.,* 1988). Engineering to increase accumulation of the osmolyte mannitol has not resulted in a level of accumulation that is sufficient to alter osmotic adjustment, but plant performance in response to stress is improved nonetheless. These overexpression studies may indicate that the alternative roles, rather than the first studied role in osmotic adjustment, may be the more important role for osmolytes.

1.2.3 Water Stress on Plants

Plants experience water stress either when the water supply to their roots becomes limiting or when the transpiration rate becomes intense. Water stress is primarily caused by the water deficit, i.e. drought or high soil salinity. In case of high soil salinity and also in other conditions like flooding and low soil temperature, water exists in soil solution but plants cannot uptake it – a situation commonly known as 'physiological drought'. Drought occurs in many parts of the world every year, frequently experienced in the field grown plants under arid and semi-arid climates. Regions with adequate but non-uniform precipitation also experience water limiting environments. Since the dawn of agriculture, mild to severe drought has been one of the major production limiting factors. Consequently, the ability of plants to withstand such stress is of immense economic importance. The general effects of drought on plant growth are fairly well known (Bray *el al.,* 1993). However, the primary effect of water deficit at the biochemical and molecular levels are not considerably understood yet and such understanding is crucial. All plants have tolerance to water stress, but the extent varies from species to species. Knowledge of the biochemical and molecular responses to drought is essential for a holistic perception of plant resistance mechanisms to water limited conditions in higher plants.

Effects of water stress

The effects of drought range from morphological to molecular levels and are evident at all phenological stages of plant growth at whatever stage the water deficit takes place. An account of various drought stress effects and their extent is elaborated below.

Successful strategies under drought stress

Classically, plant resistance to drought has been divided into escape, avoidance and tolerance strategies (Figure 2). Nevertheless, these strategies are not mutually exclusive and, in practice, plants may combine a range of response types. Plants that escape drought exhibit a high degree of developmental plasticity, being able to complete their life cycle before physiological water deficit occurs. Escape strategies rely on the successful reproduction before the onset of severe stress (Galmés *et al.,* 2007). This is important in arid regions, where native annuals may combine short life cycles with high rates of growth and gas exchange, using maximum available resources while moisture in the soil lasts. This is associated with the plant's ability to store reserves in some organs (stems and roots) which increased in droughted plants. Plants can also endure drought conditions by avoiding tissue dehydration, while maintaining tissue water potencial as high as possible, or by tolerating low tissue water potencial (Chaves *et al.,* 2002).

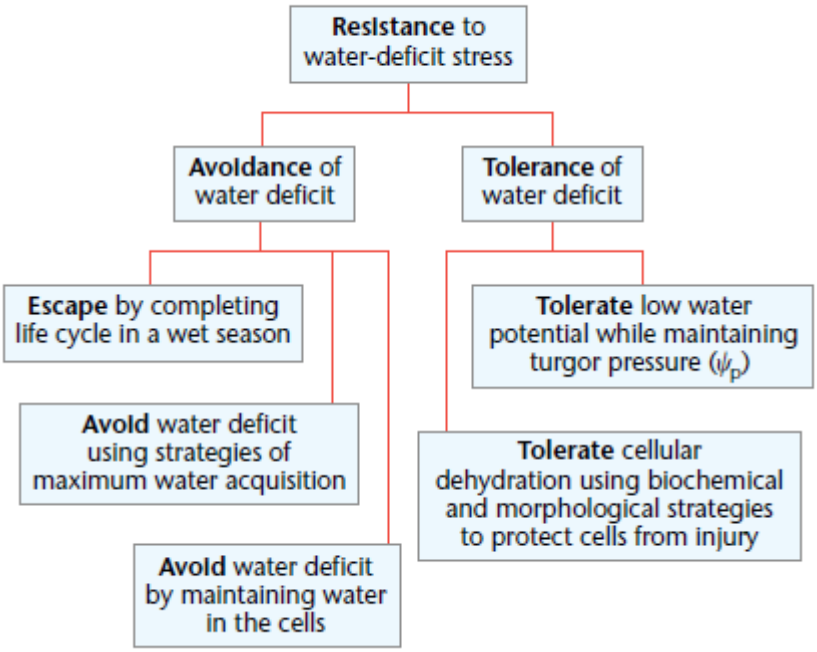

Figure 2 – Resistance to water-deficit stress can arise from mechanisms involving avoidance or tolerance of the water deficit (Bernacchia *et al*., 2004)

Dehydration avoidance is common to both annuals and perennials and is associated with a variety of adaptive traits. These involve:

(i) minimizing water loss and

(ii) maximizing water uptake.

Water loss is minimized by closing stomata; by reducing light absorbance through rolled leaves, a dense trichome layer increasing reflectance, or steep leaf angles; or by decreasing canopy leaf area through reduced growth and shedding of older leaves. Water uptake is maximized by adjusting the allocation pattern, namely increasing investment in the roots (Bernacchia *et al.,* 2004) Significant gains in crop productivity due to plant breeding for semi-arid regions resulted from enhancements in rooting depth. In addition, shedding of older leaves that contribute to water saving can be viewed as a recycling program within the plant, allowing the reallocation of nutrients stored in older leaves to the stem or younger leaves (Hirt *et al.,* 2003). During drought-induced senescence, some proteases are induced that are drought-specific and do not occur under natural aging, as is the case of some forms of cysteine proteases. Further knowledge of the regulation of these processes may allow development of plant breeding or genetic engineering strategies for the control of leaf senescence under drought. Leaf senescence, leading to short supply of sugars, is an important factor under drought stress. Therefore, delayed-senescence phenotypes may be desirable in crops where yield is source-limited and stem reserve storage and use is insufficient to support growth.

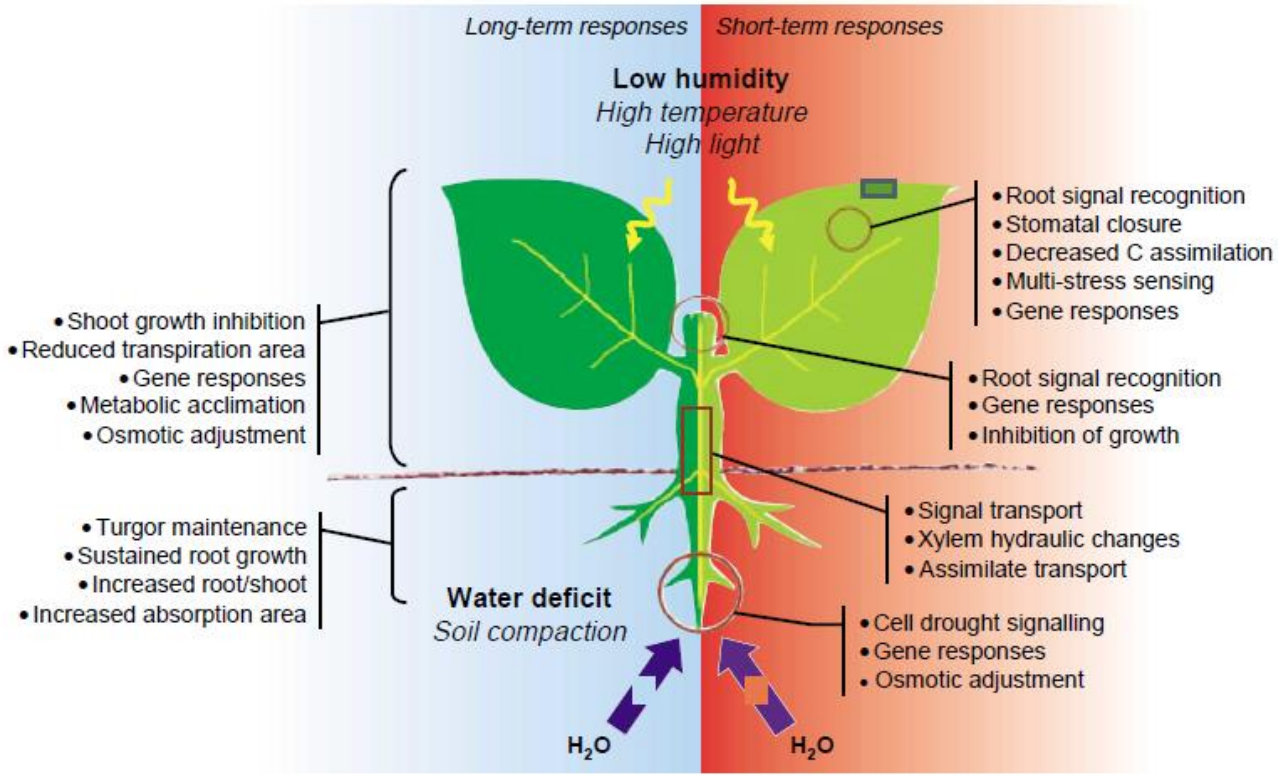

Figure 3 – Whole-plant responses to drought stress. Left, long-term or acclimation responses; right, short-term responses (Shao *et al.,* 2008).

Plant hormones and sugars are also involved in the regulation of shoot senescence. Elevated soluble sugars are known to repress the expression of photosynthesis-associated genes, in a process where hexokinases are involved in sugar sensing. One important effect of the overexpression of kexokinase is accelerated senescence (Lawlor *et al.,* 2002). Although water deficits lead to shedding of older leaves, the effects on younger leaves seem to be opposite. In fact, leaves that survive drought often show higher rates of photosynthesis and of a Rubisco content per unit of leaf area than leaves of similar age in well-hydrated plants, in what can be described as "suspension" of leaf aging. In contrast, leaves growing under drought usually reach maturity and become a carbon source at a smaller size than leaves grown in well-watered plants (Rao *et al.,* 2006).

Most of the drought-avoidance responses enable plant adjustment to low resources (water and minerals). In fact, in addition to a restriction in available water, soil drying induces a decrease in nutrients, in particular nitrogen (but also calcium), with strong interactive effects on plant growth and function. Plant adjustments to the low resources in arid environments include altered leaf structure, increased proportions of assimilates allocated to root, slow growth rates and slow organ turnover rates. Whereas short-lived organs can be discarded in response to stress, long-lived organs must optimize their resource gain (Shao *et al.,* 2008). This is the case of a change in leaf demography reported in the some desert perennial species, whose leaf lifespan doubled under drought. This response results in lower turnover rates of leaves and therefore higher nutrient-use efficiency. This also means that plants can partially compensate for lower rates of carbon gain by investing less in new leaf construction (Wang *et al.,* 2003).

However, long-lived leaves have to survive periods of hostile environment. This requires various protective mechanisms ranging from anatomical and morphological characteristics, to resist extreme climatic events, to the biochemical mechanisms targeted at dissipating excess radiant energy. A decrease in SLA may also occur in response to drought in herbaceous leaves as a result of an increased investment in structural tissues allowing increased resistance to unfavorable environmental conditions. Finally, tolerance to low tissue water potencial may involve osmotic adjustment, more rigid cell walls, or smaller cells (Shao *et al.,* 2008). Many of the evergreen shrubs and trees in arid and semi-arid regions combine the high solute concentration in living cells and low photosynthetic capacity and stomatal conductance. Small leaves are well adapted to high light and high temperature that prevail in most arid regions, because their size permits greater sensible heat dissipation and an efficacious control of water loss by stomatal closure (Figure 3).

Chemical signaling under drought stress

Stomatal closure, together with leaf growth inhibition, is among the earliest responses to drought, protecting the plants from extensive water loss, which might result in cell dehydration, runaway xylem cavitation and death. This is a common response to water deficits that develop quickly or slowly, and may result from shoot or root dehydration. The opening and closing of stomata result from changes in turgor of guard cells relative to epidermal cells. Metabolic energy (e.g. from mesophyll photosynthesis) and changes in membrane permeability are involved as well (Liu *et al.,* 2005). The detailed mechanisms of stomatal response to drought are not easy to rationalize because at any given moment stomata may be responding to a complex set of factors ranging from light intensity to CO_2 concentration in addition to leaf water status. Studies in the late 1980s showed that stomata did close in response to drying soil even when shoot water status was maintained at high turgor, either by experimental manipulation or by growing the plants with part of the root system in drying soil (split-root experiments) (Zhang *et al.,* 1991). Further evidence indicated that stomatal closure is likely to be mediated by chemical signals travelling from the dehydrating roots to the shoots. Chemical signals can be differentiated from hydraulic signals but both are important because they reduce stomatal conductance and leaf growth under conditions of water deficit. Chemical signals most probably dominate during early stages of stress before hydraulic signals are produced, and become less important under severe drought when leaf water potential declines and leaves wilt. It is likely that the hydraulic signals that may trigger the production of the hormone abscisic acid (ABA) in leaves dominate as plants become more water stressed (Holbrook *et al.,* 2002).

The production of ABA in roots and its transport to the leaves provides the plant with a mechanism for transmitting a chemical signal to report on the water status of the soil. This system could have evolved specifically for this purpose or the chemical signal could merely be a consequence of the increased production of ABA required to maintain root growth under water deficit. A dominant role for ABA in root to shoot signaling under drought and in the control of stomatal conductance was demonstrated in early reports (Thompson *et al.,* 2007). Several groups have also used a bioassay approach to show that ABA that is fed to leaves decreases transpiration (Munns *et al.,* 1992). It is also well known that ABA has a direct effect on guard cell closure. However, the ABA that acts on guard cells may not originate entirely in the roots. Recent reports suggest that the ABA that acts on guard cells could be produced in the leaves of some species, such as tomato and sunflower. In tobacco (*Nicotiana plumbaginifolia*), grafting experiments showed that ABA came from the roots under conditions of drought (Borel *et al.,* 2001). ABA is synthesized in both roots and leaves, but not much is known about the precise location of this synthesis in roots, which may influence how plants perceive and monitor soil water content. ABA

content in roots is well correlated with both soil moisture and the relative water content of roots in many plant species (Thompson *et al.,* 2007). Some reports suggest that ABA is synthesized in root tips, but synthesis has been assessed mainly by measuring ABA content rather than synthetic activity, so conclusions related to the site of synthesis need to be re-examined more carefully. One study using detached roots of pea (*Pisum sativum)* and Asiatic dayflower (*Commelina communis*) found that ABA synthesis occurred somewhere between the root tip and a point 3 cm distal to the tip (Zhang *et al.,* 1987). Recent studies have tried to locate enzymes that are involved in ABA biosynthesis, including aldehyde oxidase, which catalyzes the final steps in ABA biosynthesis; however, these studies have focused on ABA production under differing nitrogen nutrition rather than drought stress (Barabés *et al.,* 2000). More work will be required to clarify exactly where the ABA is produced in roots under drought stress. Although the role of ABA in controlling stomatal conductance is strongly supported by many experiments and experimental approaches, there may be other substances in the sap that act in concert with ABA to control drought-induced stomatal closure. For example, recent findings have suggested an interaction between jasmonic acid and ABA in plants under drought stress (Mahouachi *et al.,* 2007). In addition, the dominant role of ABA as a root to shoot signal has been challenged by experiments showing that the ABA concentrations of xylem sap from drought-stressed plants were much lower than the concentrations of exogenous ABA required to close stomata in detached leaves (Hartung *et al.,* 1998). It may be that the use of exogenous hormones in bioassays may exclude important components of xylem sap that act synergistically with ABA in planta, thus accounting for the higher levels of exogenous ABA that are required to reduce transpiration. Grafting experiments have also been used to determine the source of ABA in drought-induced stomatal closure. Some of these experiments suggest that leaf-sourced ABA is important for stomatal closure, but the influence of ABA precursors that might be transported from roots to leaves was not measured, and therefore the role of roots in chemical signaling was not completely resolved (Thompson *et al.,* 2007). Other studies show that production of ABA in the rootstock has a strong influence on and is negatively correlated with stomatal conductance (Soar *et al.,* 2006). Overall, ABA is a dominate signal in controlling growth and transpiration, but other factors could also be important. The importance and role of root-sourced ABA is still controversial, but some of the conflicting findings may be due to differences in the intensity of stress imposed and the time-course of the development of water deficit. Changes in the pH of xylem sap commonly observed under drought stress can be an important component of root to shoot signaling and may act synergistically with ABA. In many plant species (e.g. *Phaseolus coccineus* and *C. communis*), xylem sap pH becomes more alkaline when plants are water stressed and this leads to enhanced stomatal closure and even reduced growth (Ashraf *et al.,* 2007).

The potential effects of pH have been outlined previously and include:

i) changes in ABA metabolism resulting in increased leaf ABA concentration;

ii) ii) direct effects on leaf water status that could alter guard cell turgor or sensitivity to leaf ABA concentrations;

iii) iii) direct effects on ion fluxes through the guard cell plasma membrane; and

iv) iv) altered distribution of ABA in the leaf, specifically an increase the concentration of ABA in the apoplast outside of the guard cells (Figure 4).

It is this change in apoplast pH that probably has the greatest role in signaling. As ABA is a weak acid it may become protonated or deprotonated in the pH range found in the apoplast of leaves. This was clearly shown in the early 1980s in experiments on mesophyll protoplasts that documented high rates of ABA uptake at acidic pH and almost no uptake at the more alkaline pH of 7.5 (Kaiser *et al.,* 1981). In certain plant species, the pH of the leaf apoplast increases as the soil dries because of the delivery of xylem sap that is of a more alkaline pH. The ABA that arrives from roots via the xylem will remain deprotonated under the more alkaline conditions and will not be taken up passively by mesophyll cells. The result is that less ABA is transported into the mesophyll cells and a build-up of ABA in the apoplast leads to enhanced stomatal closure. Under well-watered conditions, when the apoplast is more acidic, ABA would passively enter the symplast, and apoplastic concentrations would not increase as rapidly as they do under water stress. Therefore, the effect of pH is indirect in that it leads to the accumulation of ABA in the apoplast and enhances the effect of ABA on guard cells (Liu *et al.,* 2003).

The response of xylem sap pH to drought stress does not appear to be consistent in all species or even in different experiments using the same species. One study showed that the sap pH of sunflower and *C. communis* did not change significantly as soil dried, whereas the xylem sap pH of tomato did increase as soil dried. In maize (*Zea mays*) grown using ammonium as the nitrogen source, there was no change in sap pH during early or even during later stages of drought. Other experiments using maize have been conducted in the field and have found that the sap pH of drought-stressed plants growing on a loam soil was more alkaline as the period of drought lengthened. Such inconsistencies may be explained by the fact that solute concentrations within the xylem sap vary with flow rate in intact plants (Bahrum *et al.,* 2002).

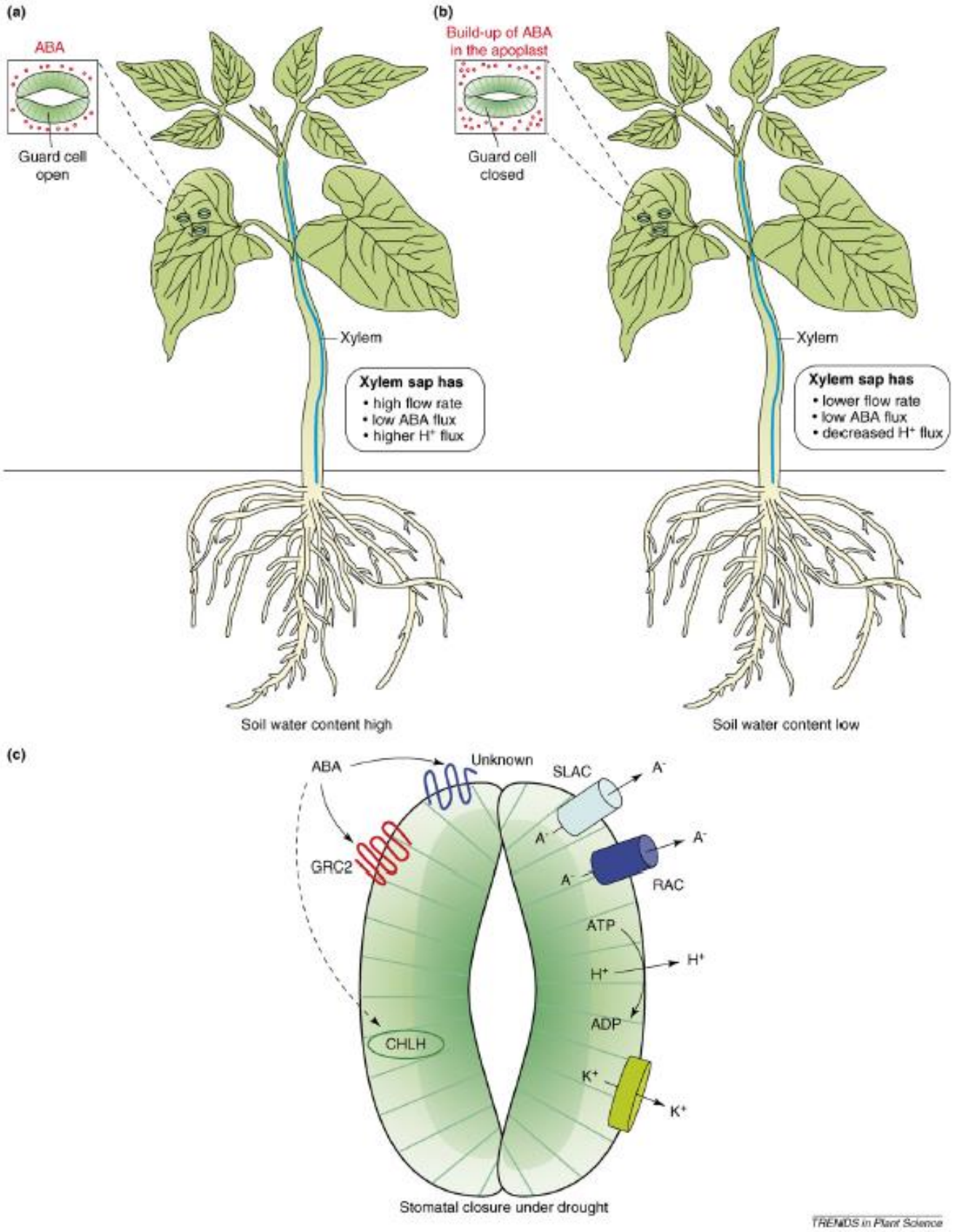

Figure 4 – Changes in xylem sap composition under drought. Drought causes the alkalinization of xylem sap pH in certain plant species. (a) Well-watered plant with apoplastic pH 6.0. (b) Plant under drought conditions with apoplastic pH 7.0 (Liu *et al.,* 2003).

Photosynthesis

Photosynthesis is particularly sensitive to the effects of water deficiency. Plants' resistance to water deficiency yields metabolic changes along with functional and structural rearrangements of photosynthesizing apparatus. Photosynthesis of higher plants decreases with the reduction in the relative water content (RWC) and leaf water potential. Lower photosynthesis rate is a usual effect of water stress in plants and has been attributed primarily to stomatal limitation and secondarily to metabolic impairment (Figure 5). However, metabolic impairment is the more complex phenomenon than the stomatal limitation though the relative importance of stomatal or metabolic inhibitions is unclear. Some studies blamed stomatal closure for the inhibition of C4 photosynthesis under water stress while others concluded that non-stomatal factors play the major role (Nayyar *et al.,* 2006).

The photosynthesis rate of leaves in both C3 and C4 plants decrease under the drought conditions. Evidence indicates that C4 photosynthesis is more sensitive to water stress and C4 plants, such as corn (*Zea mays L.*) are more susceptible to water deficiency than C3 plants, such as wheat. It explains the predominance of C4 plants in hot, arid regions - areas prone to frequent drought. C3 and C4 plants are alike in the basic process of photosynthesis like Calvin cycle and electron transport chain components, yet significant differences exist between them, which make their responses to water stress differ at a number of levels (Wilkinson *et al.,* 2004).

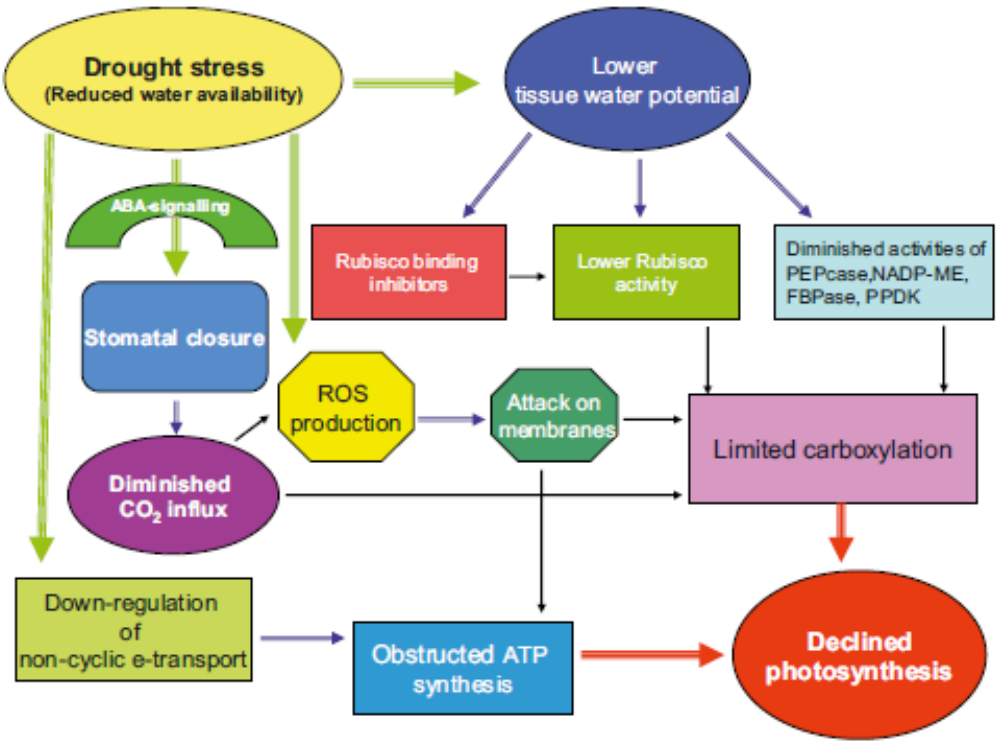

Figure 5 – Photosynthesis under drought stress. Possible mechanisms in which photosynthesis is reduced under stress (Prokic *et al.,* 2006).

There are some co-factors, which decrease plants' photosynthesis under water stress. Of them, qualitative and quantitative changes in the pool of photosynthesizing pigments, low CO_2 uptake due to stomatal closure and resistance, poor assimilation rates in photosynthetic leaves are prominent. Assimilation rates in photosynthetic leaves decreases due to reduced photosynthetic metabolites and enzymes activity, low carboxylation efficiency and inhibition of chloroplast activity at low water potential (Lawlor *et al.,* 2009). Among other co-factors of water stress, the damage of the photosynthetic apparatus through the production of ROS such as superoxide and hydroxyl radicals, worth special mention. Decrease in chlorophyll content of leaves under water stress is well known. Water stress inhibits chlorophyll synthesis at four consecutive stages:

(I) the formation of 5-aminolevuliniuc acid (ALA);

(II) ALA condensation into porphobilinogen and primary tetrapyrrol, which is transformed into protochlorophyllide;

(III) light-dependent conversion of protochlorophyllide into chlorophyllide; and

(IV) synthesis of chlorophylls a and b along with their inclusion into developing pigment–protein complexes of the photosynthetic apparatus. In the majority of cases, carotenoids are less sensitive to water stress than chlorophyll, which has been demonstrated for several species of agricultural plants.

However, unlike chlorophyll, increase in xanthophyll pigments such as zeaxanthin and antheraxanthin in plants under water stress have been reported. Xanthophyll pigments have a protective role on plants under stress, and some of these pigments are involved in the xanthophyll cycle which has inhibitory role on ROS production. Rubisco, the key enzyme for carbon metabolism in leaves, acts as a carboxylase in the Calvin cycle and as an oxygenase in the photorespiration which, however, frequently is viewed as an adverse process. Rubisco is the most critical player influencing the physiology of plants under water-stressed conditions. Under the conditions of water stress, a rapid decrease in the amount of Rubisco takes place in most plants which in turn leads to lower activity of the enzyme (Maroco *et al.,* 2002). This effect is evident in all plants studied though the extent is species-dependent. Water deficiency reduces the supply of carbon dioxide from the environment due to the closure of stomata. Consequently, photorespiration increases which ensure partial substrate replenishment and maintain the carboxylating function of Rubisco. The end result is the utilization of excess reducing equivalents in chloroplast that causes a reduction in the oxygen-free radicals' production leading to the oxidative damage in chloroplasts. The reduction in chloroplast volume can also be linked to the desiccation within the chloroplast that leads to the conformational changes in Rubisco (Miyashita *et al.,* 2005). Moreover, drought stress conditions acidify the chloroplast stroma causing inhibition to the Ru-

bisco activity. In addition, decline in Rubisco activity is also caused by the lack of the substrate for carboxylation, reduction in the amount and/or activity of the coupling factor - ATPase, loss of RBP recognition sites in Rubisco, structural alterations of chloroplasts and Rubisco, and release of Rubisco from damaged plastids. In addition to Rubisco, water stress can reduce activity of other photosynthetic enzymes to different extents such as NADP-dependent glyceraldehyde phosphate dehydrogenase, phosphoenolpyruvate carboxylase, NAD-dependent malate dehydrogenase, phosphoribulos kinase, fructose-1,6-bisphosphatase and sucrose phosphate synthase. In addition to its negative effects on dark reactions of photosynthesis, water stress also disrupts the cyclic and non-cyclic types of electron transport during the light reaction of photosynthesis. The disruption is clearer in the oxygen-releasing complex and electron transfer from protochlorophyllide to P700 (Pego *et al.,* 2000). Lower electron transport rate negatively affects photophosphorylation process and decrease ATP synthesis as well as $NADP^+$ reduction. ATPase inhibition under water deficiency is also responsible for the reduction in ATP levels in chloroplasts. All these factors cumulatively affect the intensity of photo-assimilation and the stability of the photosynthetic apparatus under the conditions of water stress. Both of the PSs in chloroplasts are affected by water deficiency, however, PS I of some plants are more severely damaged compared to PS II, though there is an opposite conclusion as well.

Incomplete or slow recovery of photosynthesis was related to oxidative stress and/or sustained photoprotection of the PSII system (Chaves *et al.,* 2002). Therefore, the analysis of PSII and electron transport chain may be expected to provide complementary and reliable information on drought-related mechanisms. The photochemical changes induced by drought/re-watering cycles can be analyzed through monitoring the chlorophyll a (Chl a) fluorescence since it was established that Chl a transients could be taken as an indicator of the water potential in the leaves and the photosynthetic status of the plants (Holbock *et al.,* 2002). Several studies provided evidence that the different phases of the Chl a fluorescence rise (OJIP) are correlated with different reduction processes of the electron transport chain; the O–J phase, for example, is thought to indicate the reduction of the acceptor side of the PSII, while the J–I phase reflects the reduction/oxidation of the plastoquinone pool (PQ), and the I–P represents the re-reduction of plastocyanin and $P700^+$ in the photosystem I (Soar *et al.,* 2006).

Oxidative damage

Exposure of plants to certain environmental stresses quite often leads to the generation of reactive oxygen species, including superoxide anion radicals (O_2^-), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), alkoxy radicals (RO) and singlet oxygen ($O^{1/2}$). Reactive oxygen species may react with proteins, lipids and deoxyribonucleic acid, causing oxidative damage and impairing the normal functions of cells. Many cell compartments produce reactive oxygen species; of these, chloroplasts are a potentially important source because excited pigments in thylakoid membranes may interact with O_2 to form strong oxidants such as O^{-2} or $O^{1/2}$ (Blokhina *et al.,* 2003). Further downstream reactions produce other reactive oxygen species such as H_2O_2 and OH^-. The interaction of O_2 with reduced components of the electron transport chain in mitochondria can lead to reactive oxygen species formation, and peroxisomes produce H_2O_2 when glycolate is oxidized into glyoxylic acid during photorespiration.

1.2.4 Water uptake by roots: effects of water deficit

Fewer efforts have been made to investigate the input side of the water balance, i.e. the acquisition of water from soil. It has been known for a long time that the hydraulic conductance of roots is variable. It depends on factors such as water shortage and salinity of the soil and on the demands for water from transpiring shoot. Other important factors are nutrient deficiency, anoxia, temperature, and heavy metals. The lack in current knowledge of how these factors influence water supply is largely due to technical problems in measuring, unsufficient detail, the hydraulic architecture of roots. Basic hydraulic properties of roots are not yet adequately understood, simple because roots in the soil are much less accessible than shoots.

In a schematic way, it is shown in Figure 6, how the variable root hydraulic resistance would contribute to the water potencial (water status) of the shoot. Usually, the vapour pathways in mesophyll air spaces, stomatal pores, and the boundary layer outside the leaves represent the highest hydraulic resistance in the soil-plant-air continnum (SPAC), and most of the water potencial difference between soil and atmosphere will drop here. At a given water potencial difference ($\Psi_{soil} - \Psi_{atm}$) and water flow across the SPAC, the water potencial of the shoot may be dominated by the drop in water potencial across the root hydraulic resistance which is usually the highest within the liquid part of the SPAC. In other words, to improve the water status of the shoot, the plant could either increase stomatal or decrease root hydraulic resistance; the latter does happen (besides the regulation of transpiration) (Hose *et al.,* 2000). The hydraulic resistance of

the xylem is relatively small. It way, however, become important under conditions of stress, when high tensions in the xylem cause cavitation and interrupt water flow in vessels between root and shoot.

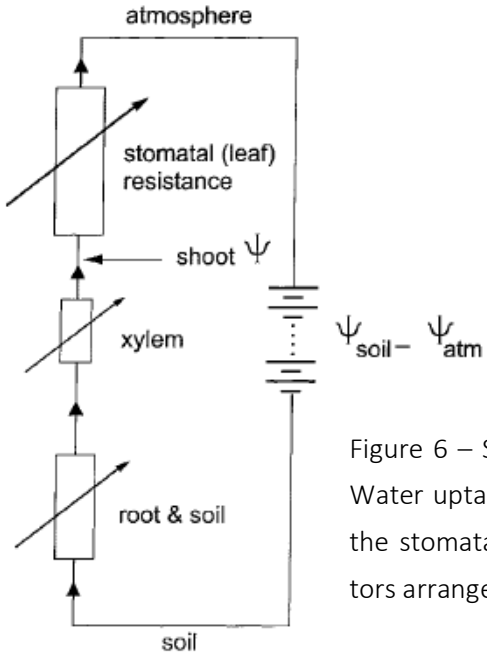

Figure 6 – Simplified model of steady water flow across a plant. Water uptake by roots, long-distance transport in the xylem and the stomatal resistance are denoted by variable hydraulic resistors arranged in series (Hose *et al.,* 2000)

Instead of actively taking up water, roots just allow it to pass through them in response to water potencial gradients usually set up by transpiration. The complex anatomical structure of roots results in a complex pattern of water flow. Transport across various tissues (epidermis, cortex, stele) has to be considered during the radial passage across the root cylinder as well as a longitudinal flow component (axial transport in xylem vessels). During stress, the anatomy of root tissue will change, largely, because of stress (such as water deficit) induces the development of apoplastic barriers for water and ion flow. Their formation represents a fundamental adaptive strategy of plants to survive in and adverse environment. In addition, the hydraulic conductivity of root cell membranes may be reduced due to a closure of water channels or aquaporins in root cell membranes (Harvengt *et al.*, 2000). So, besides transport across the apoplast, there is some water flow across cell membranes. Water deficits reduce root growth and cause a pronounced suberization of the apoplast and, perhaps, also affect the cellular passage (water channels). This influences the water balance by reducing the capacity of roots to take up water (Henzler *et al.,* 2004).

Root water uptake capacity is the consequence of intrinsic root hydraulic properties of a given plant. Water can flow from soil to xylem vessels following three different pathways: apoplastic, symplastic, and transcellular ones (Figure 7). The apoplastic path includes the water circulating along cell walls and intercellular spaces. The symplastic path comprises the water circulating along cell walls and cells through plasmodesmata. Finally, the transcellular path consists of the water flowing across the cell membranes (plasmalemma and tonoplast). Symplastic and trascellular paths cannot be distinguish experimentally, so the sum of both pathways is called cell-to-cell pathway (Holbrook *et al.,* 2003).

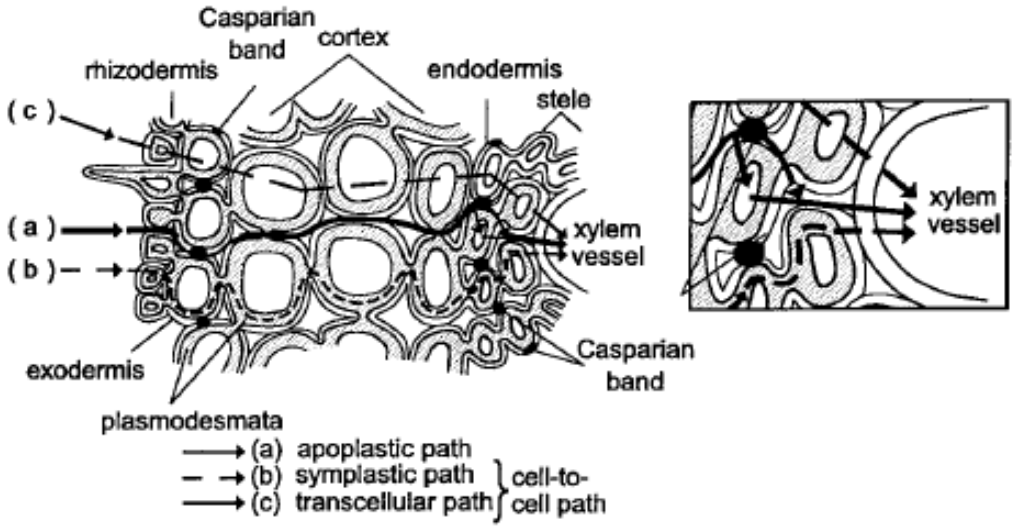

Figure 7 – Transport pathways in roots (Holbrook *et al.,* 2003).

At the same time, there is an exchange of water among the three pathways depending on the environmental conditions. Thus, under non-stressful conditions water circulates mainly through the apoplastic path due to hydrostatic forces. Conversely, when transpiration is restricted at night or under stressful conditions water flows predominantly through the cell-to-cell path because of the osmotic forces (Henzler *et al.,* 2004). Under some circumstances, it has been found that water flows only via cell-to-cell path. Some roots develop exo- and endodermis, which restrict the water flow through the apoplastic path, and therefore water has to cross cell membranes to reach the xylem vessels and consequently it has to flow through aquaporins. Aquaporins are membrane intrinsic proteins that allow the passage of water and other small molecules through them always following an osmotic gradient.

An important characteristic of a drought resistant species is an extensive and widely spreading branching root system. Independent of the modified rooting patterns and water uptake capacity and efficiency, it is reported that grain sorghum roots are still active and extract water even when soil water potential goes below −1.5 MPa while in maize embolism occurs at around −1.6 MPa (Cochard *et al.,* 2002). In general, under drought conditions grain sorghum develops a deeper and more evenly distributed root system than maize. However, found that rooting characteristics of various improved sorghum lines differed significantly, then their functioning, such as water uptake and root water uptake efficiency, may also vary widely. Compared with a drought susceptible cultivar, for example, a drought tolerant sorghum cultivar showed higher water uptake efficiency, fewer late metaxylem vessel per nodal root, smaller leaf area, and well developed sclerenchyma (Schreiber *et al.,* 2001).

Drought Effects on Root Morphology and Anatomy

The most common effect of drought stress is an increment of the root:shoot ratio. Such a change in the proportion of root biomass with respect to shoot biomass can be caused by a reducing in shoot growth without any change in root growth, by a direct investment in root growth, or because root growth is less restricted than shoot growth membranes (Harvengt *et al.*, 2000). The increase of root: shoot ratio under drought conditions has been as a mechanism to explore more soil volume in order to absorb water from deeper soil layer, which is not available for less developed roots. However, no clear relationship between root:shoot ratio and drought tolerance has been always observed. This lack of correlation can be explained because there are differences in water uptake capacity in the root system. It was observed that under drought conditions *cowpea* roots increased the capacity of absorbing water only of the deeper younger roots. Similar results were found, in other study, in maize plants. At the same time, a better correlation between root:shoot ratio and drought tolerance has been observed when the shoot part was expressed in terms of area instead of weight. In addition, root length or root length density under drought conditions have been correlated with water stress tolerance (Schreiber *et al.,* 2001). A drought tolerant variety of maize had longer nodal roots than a sensitive variety under drought conditions. Furthermore, was observed a correlation between root density in deeper soil layers and yield in different peanut genotypes with contrasting tolerance to pre-flowering drought stress. However, although it could be a relationship between root growth under drought conditions and drought tolerance, it is clear that this parameter alone cannot explain the higher capacity of water absorption of such roots. Thus, when comparing maize versus rice, it was found that maize plants were able to absorb more water from deeper soil layers under severe drought conditions than rice, and this was not only explained by a better root development of maize plants, but also by a higher water uptake capacity per unit of root surface. So, there must be intrinsic properties of root tissues that make a particular root more efficient in terms of water absorption under drought conditions than others (Stasovky *et al.,* 2002).

Roots may develop apoplastic barriers to water and solutes from just 10 mm from their root apexes to their basal zones. These barriers may consist of suberin layers deposited in the inner parts of the cell walls of endo and/or exodermis, lignin depositions in sclerenchyma and stellar cells, and casparian bands between exo and/or exodermis cells. It is well known that these barriers increase under drought conditions and can diminish root water uptake capacity estimated as root hydraulic conductivity (L) (Stasovky *et al.,* 2002).

1.3 ARUNDO DONAX

Arundo donax (giant reed cane) is an invasive perennial weed grass plant belonging to the *Poaceae* family of the A*rundinae* tribe, widely found in subtropical and warm temperate regions all over the world. The success of its diffusion is due to its spontaneous propagation by rhizome fragmentation and sprouting from the cane nodes, but also mostly, to its cultivation. In fact, the canes are used for many purposes such as roof thatching, fishing rods, reeds in woodwind instruments, etc. It is one of the largest herbaceous grasses and even though *A. donax* is a C3 plant, it shows high photosynthetic rates and unsaturated photosynthetic potential in comparison with C4 plants. The origin area of giant reed is still a matter of debate because biogeographic and evolutionary origin of this species has been obscured thought ancient and widespread cultivation (Angelini *et al.,* 2005). There is no agreement on the localization of the area where it originated. Botanical and historical evidence support the hypothesis that the origin (semi-domesticated or at least incipient) started from a pool of wild plants native in the Mediterranean region. However, giant reed has been cultivated in Asia, Southern Europe, North Africa and the Middle East for thousands of years. It was used to satisfy local necessities, such as training stakes, baskets and mats, walking sticks and fishing canes. In Italy, this species has been used industrially since 1930, when a company registered a trade mark to obtain cellulose past for the production of rayon viscose and paper (Ceotto *et al*., 2010), although it is also considered an invasive and dangerous weed plant in several countries. Despite these problem, in recent years *A. donax* aroused the interest of the scientific community because of its high potencial in EROEI value (energy returned on energy invested) compared to other energy crops. This fact is due to its great biomass productivity and that this species is also cropped in low input conditions (Angelini *et al.*, 2009). If we consider that the length of cropping life of *A. donax* lasts about 12 to 15 years, without irrigation (with the expection of the first year), with low fertilization (this question needs further research due to the scarcity of experimental data), without phytosanitary and weeding treatments (Angelini *et al.*, 2005), it's easy to understand the reason why the interest of the crop has significantly aroused (Figure 8).

According to its fast growth rate and ease of vegetative propagation, giant reed is considered as invasive species in the warm-temperate regions with winter floods that widely disperse this plant (Pilu *et al.,* 2012). However, contrary to its invasive characteristics, as in the USA, giant reed has received social consensus by Mediterranean farmers, who have learned to live and deal with this species a long time ago due to its multiple usage. Indeed, giant reed is naturalized in the Mediterranan basin, it grows spontaneously and abundantly all over Mediterranean and warm-temperate areas of the world (In addition, thanks to a highly developed canopy and a deep root

system, it covers the whole ground in the years following the establishment preventing the risk of soil erosion (Cosentino *et al.,* 2005).

1.3.1 Cultivation and Production in *Arundo donax*

It was reported an average aboveground biomass in *Arundo* of 39.6 Mg dry matter ha^{-1} in a 7-year study conducted in the Low Po Valley (Northern Italy) and reported an average yield of 37.7 Mg dry matter ha^{-1} in a 12-year study carried out in the coastal Tuscany (Central Italy) (Angelini *et al.,* 2008). Giant reed growth rates from established rhizomes in California, averaged 6 cm per day in the first 40 growning days and 2,5cm per day in the first 150 growning days. Giant reed can grow more than 5 cm per day under optimal conditions.

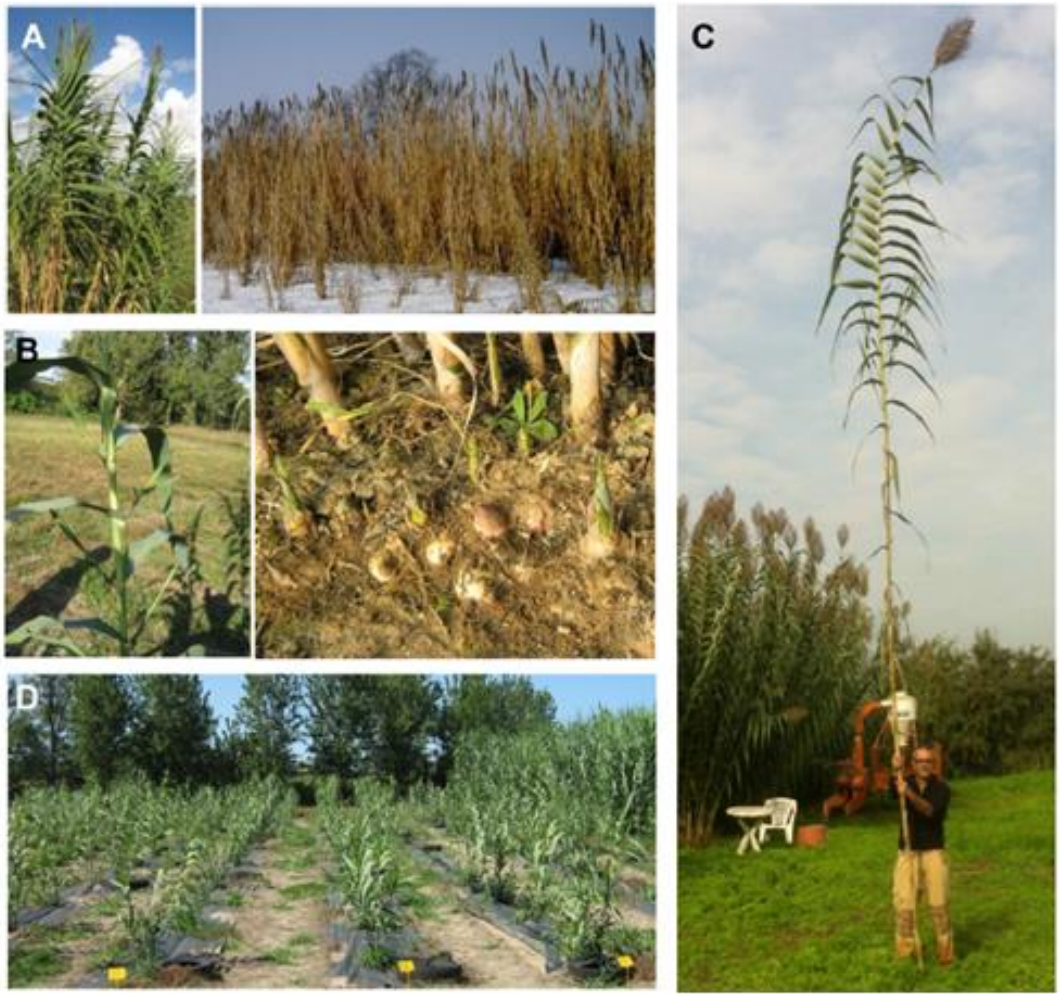

Figure 8 - Life cycle, propagation and culture of *A. donax*. (A) Mature plants in summer (left) and in the winter (right). (B) Propagation by shoots sprouting from canes (left) and from rhizome (right). (C) Single cane, reaching the height of 8 m (D) Culture of *A. donax* by cane cuttings propagation (using mulch) (Mariani *et al.,* 2010).

Dormant giant reed is able to survive temperatures as low as 0°C, but it can suffer severe damage by frost at the seedling stage or during spring re-growth. Sufficient moisture is needed after planting in the first year in order to assure a good stand that may last an average of 10 years (Mariani *et al.,* 2010). Weed growth can be reduced by lack of moisture during its first year but drought does not cause usually damage to stands which are two- to three years old. Giant reed can overcome dry growing conditions and still produce yields of up to 20 tons ha^{-1} dry biomass, although with moderate irrigation it may produce larger yields (Christou *et al.,* 2000).

1.3.2 Interest as a Bioenergy Crop

From the energy point of view, *A. donax* can be used to produce energy by direct combustion or to produce second generation biofuels, such as bioethanol, generated by alcoholic fermentation of lignocellulosic biomass pretreated to facilitate sugar release (Christou *et al.,* 2000). Of course, the pretreatment processes used to increase carbohydrate degradability (e.g. steam explosion, acid and heat treatment) consume energy. For this reason, a goal of the genetic improvement of *A. donax* and other grasses should also consist in the modification of cell wall composition in order to facilitate the use of the lignocellulose products in the biomass. The presence of phenolic compounds in the cell wall limits the carbohydrate degradability and consequently the energy value. The lignins, which are tightly associated to cell wall carbohydrates, thus prevent physical access of enzymes to cellulose and hemicelluloses and strongly limit their enzymatic hydrolysis. Cross linkages between lignins and arabinoxylans, and between arabinoxylan chains, through ferulate and diferulate bridges, also greatly impede cell wall degradability (Scordia *et al.,* 2012). Although *A. donax* is one of the tallest herbaceous plants, its lignin content is similar to that of other grass species, with an average value equal to 21%, while it equal to 19% in switchgrass, 20% in sugarcane, and 23% in *miscanthus*. The latter values are however still higher than in maize, with lignin content close to 11%. Lignins of *A. donax* are composed of guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) units with an S/G ratio of 1.13 - 1.32 which is a little lower value than in maize. In fact, it was showed that little differences were noticed, for example, between sorghum straw and *A. donax* capabilities to produce ethanol after acid pretreatment and enzyme hydrolysis using *Zymomonas mobilis*. In another work, *A. donax*, elephantgrass (Pennisetum purpureum), miscanthus and sugarcane were compared for their capacity to produce bioethanol using cellulose solvent-based lignocellulose fractionation (CSLF) pretreatment and enzymatic (cellulase) hydrolysis (Ge *et al.,* 2011). The results obtained showed no significant differences among these energy crops, consequently indication that one of the most important parameters to be considered to optimize the EROEI value is the biomass production per hectare. Of

course, another promising way to use these energy crops is through the direct anaerobic digestion of chopped biomass to produce biogas without any pretreatment. However, for both bioethanol and biogas production, a higher susceptibility of the biomass to pretreatments will allow the use of more environmentally friendly processes, lowering pollution and energy costs.

1.3.3 *Arundo donax* as a candidate plant for energy production

Taken together, these data suggest that *A. donax* could be considered a good candidate to supplement/replace maize, sorghum, and other energy crops (Table 1) used in particular to feed anaerobic digesters to produce green energy in Northern Italy (Schievano *et al.,* 2012). *A. donax* could thus be considered as the best candidate for cultivation in those Mediterranean areas characterized by 1800°C GDD (growth degree day) and useful rainfall equal at least of 300mm during the season. Another promising perennial energy crop that could be utilized is *Miscanthus* (*Miscanthus x giganteus*) a sterile, triploid interspecific hybrid, also needing vegetative propagation by rhizome fragmentation or in vitro cultures.

However, a comparison of *A. donax* and Miscanthus in a long-term field experiment (12 years) in Central Italy (Pisa) showed a higher biomass production in the case of *A. donax* with an average biomass production of 37.7 tons of dry matter per hectare and 28.7 t ha^{-1} for *Miscanthus*. These results have been obtained without irrigation and by fertilizing every year with 100 kg P_2O_5 ha^{-1}, 100 kg K_2O ha^{-1} and 100 kg N ha^{-1} (Angelini *et al.*, 2009). In another work conducted in Southern Italy by a lower level of biomass production (22 t ha^{-1}) was reported for *A. donax*, probably due to the scarcity of rainfall (Cosentino *et al.,* 2005). Hence, in Northern Italy where there is often a high water table and a good summer rainfall, *A. donax* could be cultivated without irrigation (apart from the first year) and with a minimum or no chemical fertilization. Here we can consider the possibility to use instead as fertilizer the manure/digested matter that are considered as wastes of the agriculture in this region rich in cattle rearing and digesters.

Table 1 - Comparison among maize, sorghum and *A. donax* as energy crops (Angelini *et al.,* 2009)

	Fresh matter yield (t ha^{-1})	Dry matter (%)	Dry matter yield (t ha^{-1})	Culture cost (Euros ha^{-1} $year^{-1}$)
Maize	70	30	20	2,100
Sorghum	128	15	19	1,700
A donax	85 - 130†	42	36 - 55	700‡

†85 t ha^{-1} in marginal lands and 130 t ha^{-1} in fertile lands
‡considering a 15 years duration of the crop

1.3.4 *Arundo donax* Water Use Efficiency

Although *A. donax* only reproduces vegetatively it has greatly expanded its native range and is currently listed as an invasive species throughout the Sunbelt of the United States and Mediterranean regions world-wide. Identifying fundamental physiological interactions between *A. donax* and its environment is paramount to effectively forecasting the consequences of *A. donax* cultivation. Despite many claims supporting or condemning the use of *A. donax* as a bioenergy crop, basic questions about this species' ecophysiology have been, heretofore, unclearly demonstrated. Of critical need in semi-arid Mediterranean regions is how much water might be consumed per unit of biomass gain (Mann *et al.,* 2012). Answering this question is essential for agronomists who claim great biomass production with minimal resource requirements, and for ecologists who predict reductions in water resources should *A. donax* escape cultivation, especially given the unpredictability in quantity, quality, and timing of freshwater resources in a changing climate. In the case of Giant reed and *Miscanthus*, the use of water resources seems to be one of main aspects which differentiates the two species: giant reed can survive under very wet or dry conditions for a long period, while *Miscanthus* does not tolerate prolonged drought and responds to water stress by senescing, losing leaf area and increasing root growth with respect to rhizome growth (Lewandowskiet *et al.,* 2003). In addition, giant reed is a C3 species, while *Miscanthus* is a C4. It is known that C4 plant species tend to be more efficient in their use of water because they avoid photorespiration, while C3 species need more water than C4 species to produce the same amount of biomass. It is know that *A. donax* is considered a drought-resistant plant. One of the characteristics of a drought-resistant plant is the deep, wide-spreading, branching root system. Deep rooting of annual food crop species has been extensively studied before (Araki *et al.,* 2000).

Under drought stress, water use efficiency (WUE) is the main concern rather than the absolute production. Several studies have demonstrated that the WUE of sweet sorghum is higher than that of maize, grain sorghum, and other C4 crops and that it changes in function of the timing and intensity of the drought stress (Liakatas *et al.*,2007). WUE was either maintained or slightly increased when drought stress occurred at later growth stages while with an early stress WUE decreased by about 20%. Since the ability to take up water from deep soil layers and WUE seems to operate independently on drought tolerance (Passioura *et al*. 1998), drought-tolerant *Arundo donax* species may have either one or both of these traits but not necessarily both of them, however, information on that regard is not available. There is no quantitative information on the ability of the deep roots of *Arundo donax* to extract water from wet subsoil layers when top soil is prone to drought. For giant reed, it was reported cumulative evapotranspiration values be-

tween 1000 and 1500 mm year^{-1} under non-limiting water conditions, while it was estimated values close to 1000 mm year-^{1} in *Miscanthus*, against c.a. 600 mm year^{-1} in maize.

1.4 PURPOSE OF THE STUDY

Giant reed (*Arundo donax L*.), is one of the most promising energy crop because of its high yielding potential in a wide range of habitats, especially in the arid an semiarid areas of the Mediterranean region. In such areas it is common to find large amounts of water accumulated in deep layers. Despite that, many crops suffer from severe stress due to the scarce and erratic rainfall, suggesting that deep water sources are underutilized. Giant reed is a deep rooted crop and can survive prolonged dry periods (Lewandowski *et al.,* 2003) Therefore it's great drought resistance could be related to its capacity to develop deep roots, but specific information on the functioning or root distribution is missing. Such information may provide new insights into the mechanisms involved in soil water uptake and drought resistance of *A. donax*. Besides that it is hypothesized that *A. donax* has the capacity of either adjust its water uptake strategies and root distribution in function to water availability or adjust its stomata conductance to maintain its physiological functions. However, information on the independent or coordinated functioning of both mechanisms is missing. It is well known that root signals such as Abscisic acid (ABA) alter the osmotic potential of stomatal guard cells, leading to stomata closure, but it is not that well know to what degree such hormonal signals could alter the root architecture. Establishing a direct relationship between the location and timing of ABA biosynthesis is difficult, especially in deep rooted crops. Therefore determining if the roots present in dry topsoil layers could function as an overriding ABA biosynthesis location when deep roots has readily access to water, or vice versa could help to develop more efficient water use strategies or improved drought resistance in *A. donax*. Beside that a better understanding of the real importance of deep roots could be elucidated. The analysis of the hormonal and physiological interactions of *A. donax* under alternating optimum-drought conditions, however, requires the setup of a system that allows controlling independently the water sources, its levels, root distribution through the soil profile, and canopy growth monitoring. Besides that such system may allow to validate water uptake and root growth models. Even though the recent technological advancements allow the study of deep roots, it remains elusive and costly, especially under field condition where many uncontrolled factors intervene. Most of the current technologies used for study roots in laboratory were developed for small soil volumes and shallow layers (e.g. transparent window interfaces) therefore they need to be adapted to the specific needs to study deep roots.

2. METHODOLOGICAL APPROACH

2.1 EXPERIMENTAL SETUP AND GROWTH MEASUREMENTS

Giant Reed (*A. donax*) was grown at an experimental greenhouse under controlled environmental conditions (with average minimum and maximum temperatures of 25 °C and 37 °C) at Bologna University (44°33'N) in twelve cylindrical transparent rhizotrons. The twelve cylindrical transparent rhizotrons of 1 m height and 30 cm diameter were placed in a greenhouse under controlled environmental conditions at Bologna University. In order to create a top-bottom split root system, each rhizotron was compartmentalized into two layers of about 45 cm each with an impervious *Petrolatum* layer to water but permeable to root growth. In order to do that, the minimum root impedance by the *Petrolatum* layer was determined. That is, a petroleum jelly layer that do not affect the normal root growth in depth was determined (Figure 9, c). In each compartment, independent subsurface drip irrigation systems and soil moisture sensors (*Spectrum s100*) were installed for constantly monitor and adjust the soil moisture content to predetermined values. Each compartment of the rhizotrons was filled with sandy loam soil to a bulk density of about 1.3 g cm^{-3}. Transplanted local *A. donax* seedlings (Figure 9, a) were grown under well-water conditions until roots reached the bottom compartment. The seedlings were reproduced in vitro by a nearby nursery then they were transplanted in small pots which contained sandy loam soil, after a couple of months they were definitively transplanted in the rhizotrons which contained the same soil as the small pots. Thereafter, in half of the rhizotrons two contrasting water levels with a specific isotopic signature were imposed: drought at shallow layers (treatment - between 10 and 6% v/v) and field capacity at deep soil layers (control - 23% v/v) (Figure 10). Drought stress was imposed to topsoil layers at 95 DAT (Days after transplanting). Aboveground plant biometric measurements were taken weekly, starting one week after transplanting. A tiller identification system was developed and the height of all stems was measured from the ground level to the insertion point of the last fully developed leaf. Stem diameter was determined at 1 cm above soil surface. Number of tillers and leaves were also recorded.

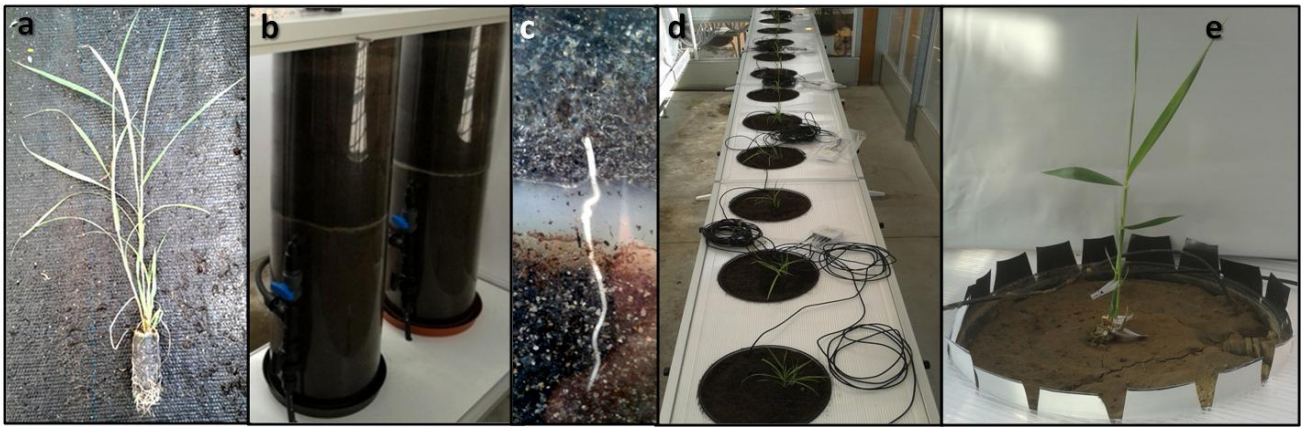

Figure 9 - (a) Micropropagated local ecotype seedling of *Arundo donax* L.; (b) Cylindrical rhizotron-system; (c) Root permeable layer; (d) Whole system after transplanting; (e) Well established seedling.

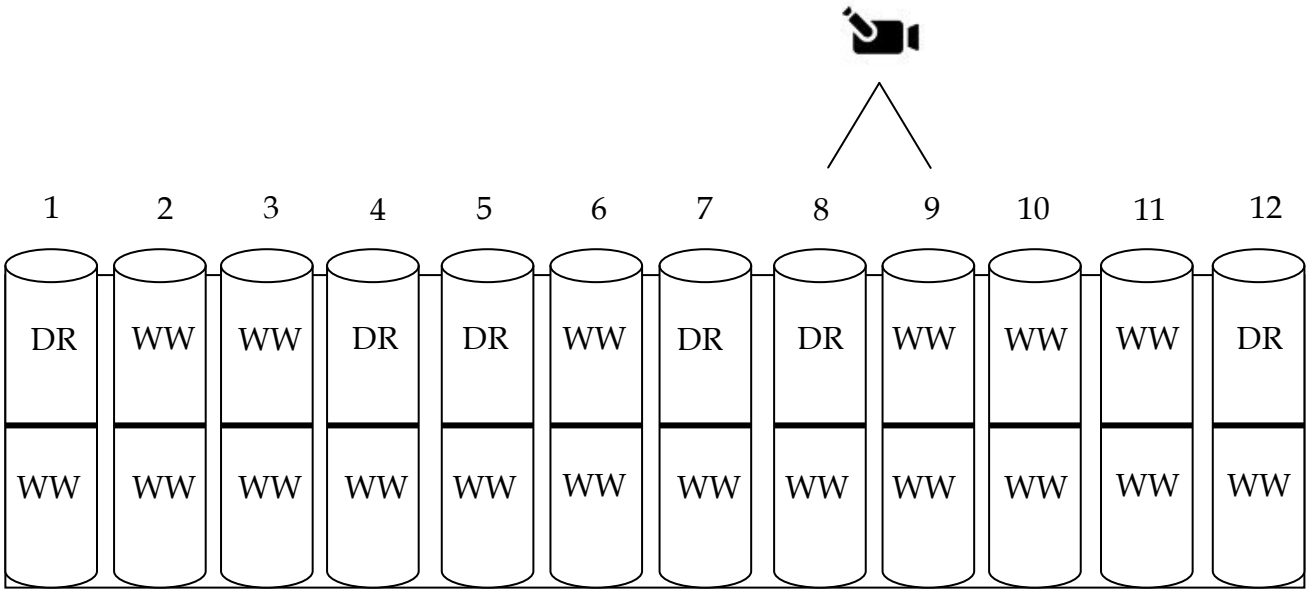

Figure 10 – Representative scheme of the experimental setup. The twelve rhizotrons have two contrasting water levels with a specific isotopic signature imposed: drought at shallow layers (treatment - between 10 and 6% v/v) and field capacity at deep soil layers (control 23% v/v). WW- Well-watered; DR- Drought. The rhizotrons 8 and 9 were used for following the growth with a video camera.

2.2 LEAF RELATIVE WATER CONTENT

An advantage in characterizing plant drought stress by relative water content (RWC) is that it reflects the dynamic water balance between water flow into and out of the tissue. RWC is the ratio of the leaf water content com-pared to the fully hydrated conditions. Leaf discs collection was started at 45 days after initiated the treatments and thereafter biweekly. Leaf discs were bagged in pre-moistened, re-sealable, plastic bags and brought to the lab in less than 30 min after harvesting to pre-vent desiccation. The leaves were then individually weighed to obtain its fresh weight. The fresh leaves were returned to the plastic bags that were subsequently filled with deionized water, and sealed. The leaves were soaked for 18 h at which point they were removed from the plastic bags, surface dried by blotting with a paper towel and then re-weighed. After the fully hydrated leaf discs weights were recorded, the leaves were dried at 105°C for 24 h in drying oven. The oven-dry leaf mass was weighed and recorded. RWC was calculated using the formula: RWC (%) = [(FW − DW)/(TW − DW)] × 100, where FW, DW and TW are fresh, dry and turgid weight, respectively (Figure 10 b,c).

2.3 LEAF GAS EXCHANGE AND FLUORESCENCE

Assimilation and transpiration rates were assessed by leaf gas exchange measurements by a portable gas analyzer (CIRAS-2, PP Systems) on the youngest fully expanded leaf, near the middle part of the leaf blade on its adaxial face, of the youngest and oldest tillers. Leaf gas exchange was always taken at the same daytime (between 9.00 to 12.00 am). Measurements were started one week before initiating the stress treatments, thereafter measurement were taken every two weeks. The environmental conditions set for the measurements were PAR1400 $\mu molm^{-2}$ s^{-1} and 380 $\mu mol\ mol^{-1}$ of CO_2. The CIRAS-2 produced measurements for photosynthesis (Pn), transpiration (E), stomatal conductance (g_s) and the differences between atmospheric (C_a) and internal CO_2 concentrations (Ci) (Figure 11 d,e).

Together with leaf gas-exchange measurements, plant vitality was assessed through leaf florescence measurements. Plant vitality was characterized by the performance index, an integrative parameter that includes: the density of fully active reaction centers; the efficiency of electron movement by trapped exciton into the electron transport chain beyond the primary quinine electron acceptor (QA); and the probability that an absorbed photon will be trapped by the reaction centers. In contrast to FO, FM, and other derived parameters commonly used to analyze the fluorescence signals, the performance index is more sensitive to drought stress and therefore in the present study such index is used in lieu of plant vitality. Chlorophyll a fluorescence transients were measured by a Plant Efficiency Analyzer (Handy PEA, Hansatech, UK) with an initial time

resolution of 10 µs. The transients were induced by a red light with peak wavelength of the emitted light at 650 nm. Data acquisition was every 10 µs for the first 2 ms and every 1 ms thereafter (Strasser *et al.,* 2000). In order to generate maximal fluorescence and the closure of all the reaction centers (RCs) in all treatments, the light intensity was set at 3000 µmol photons m^{-2} s^{-1}. Fluorescence measurement was taken continuously throughout the trial, started one week before initiating the stress treatments, thereafter measurement were taken every two weeks. Prior to measurements selected young fully developed leaves of the youngest and oldest tillers, per treatment were adapted to darkness for 20 min using appositely done leaf-clips. All measurements were taken on the adaxial surface of the selected leaves. The potential quantum yield measured in dark-adaptedleaves (Fv/Fm) is a ratio of the maximal fluorescence (Fm) minus the resting or null fluorescence (Fo) divided by the maximal fluorescence, and was calculated automatically by the CIRAS-2. The JIP-test (Strasser *et al.* 2000) was used to analyze the Chl a fluorescence transients and the following original data were acquired: maximal (Fm =P) and minimal (Fo =O) measured fluorescence intensity. From these traits, relationships between the biochemical reactions occurring in PSII and specific JIP-test parameters could be established. The Fo was taken at 50 µs when all RCs are open. Besides that, the time resolution of the Handy PEA allows to determine two intermediate steps in the fluorescence transient; the J step that shows up at about 2 ms and the I step that shows up at about 30 ms. The OJIP transients were plotted in logarithmic time scale in order to make visible all the aforementioned steps. The performance index (PI) was calculated as function of its three independent components: the density of active RCs (RC/ABS), trapping probability (P[TR]) and electron transport probability (P[ET]). Table 2 shows a number of derived parameters from the original fluorescence data acquired that are most commonly used to evaluate the PSII function. All the calculated values refer to time zero (onset of fluorescence induction).

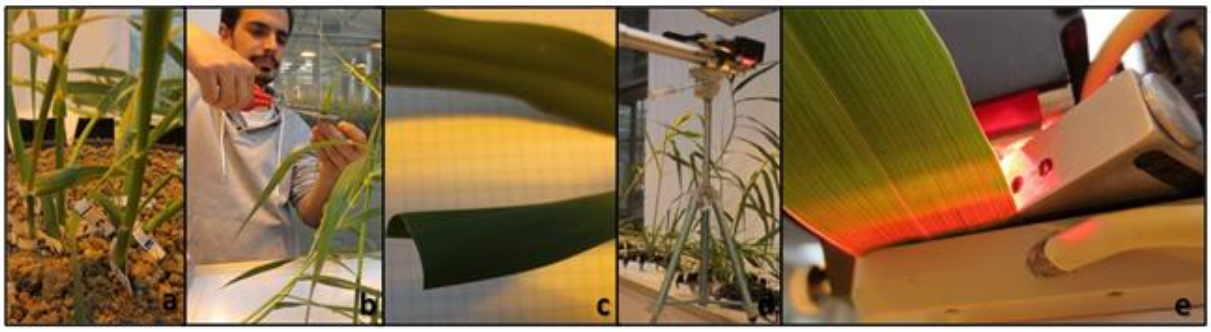

Figure 11 - (a) Tiller identification system, (b,c) Relative water content sampling; (d,e) Leaf gas exchange measurement.

Table 2 - Summary of some derived parameters from fast fluorescence transients used in the OJIP test (Monti *et al.,* 2013)

Parameter	Description
Extracted and technical fluorescence parameters	
F_o	Fluorescence intensity at 50 μs
F_m	Maximal fluorescence intensity
V_J	Relative variable fluorescence at 2 ms: $(F_{2ms} - F_o)/(F_m - F_o)$
$(dV/dt)_o$ or M_o	Net rate of PSII closure: $4(F_{300\mu s} - F_o)/(F_m - F_o)$
Flux ratios or quantum yields	
φP_o or TR_o/ABS	Trapping probability or maximum quantum yield of primary photochemistry: $(1 - F_o)/F_m$ or F_v/F_m
φE_o or ET_o/ABS	Electron transport efficiency: $\varphi P_o(1 - V_J)$
PI_{ABS}	Performance index: (RC/ABS) (F_v/F_o) $[(F_m - F_{2ms})/(F_{2ms} - F_o)]$
RC/ABS	Contribution to PI_{ABS} of the active reaction center density: $(F_{2ms} - F_o)/4(F_{300\mu s} - F_o)$ (F_v/F_m)
(F_v/F_o)	Contribution to PI_{ABS} of the trapping probability (P[TR])
$(F_m - F_{2ms})/(F_{2ms} - F_o)$	Contribution to PI_{ABS} of the electron transport probability (P[ET])

2.4 SOIL WATER CONTENT, ROOT WATER UP TAKE EFFICIENCY

Soil moisture was estimated using soil moisture probes (*EC-5 Decagon Devices*) that were installed at about 0.25 and 0.75 m depth in the center of each tube. The probes were calibrated against volumetric water content (gravimetric method) measurements taken through a wide range of soil moisture contents. In order to calculate the irrigation needs soil moisture readings were taken continuously throughout the experimental period using the aforementioned moisture probes and automatic data loggers which recorded the readings as 6-hour averages.

Roots growth was monitored from the start of treatments through root drawings on transparent sheets. Every two weeks, visible roots were traced with different color markers. The root drawings were scanned and then root length was analyzed with specialized software for image analysis (ImageJ). Root length density (RLD) and root growth rate was derived from the increments in root length with time (Figure 12).

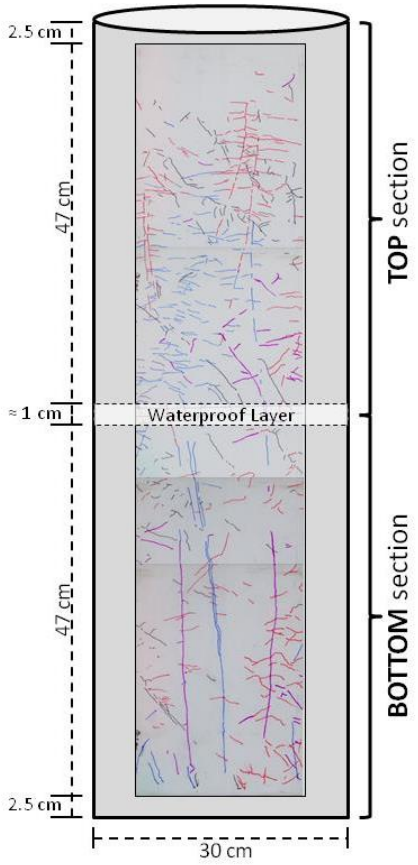

Figure 12 - Schematic representation of root sampling. Roots were traced with different color markers (e. g. black, red, blue, etc.) on successive dates.

2.5 ABSCISIC ACID ASSAY

Leaf samples along the stems from the youngest and oldest tillers were taken biweekly and frozen at -26^{o}C for further analysis. Prior to the assay *Arundo donax* leave samples were lyophilized for 48 hours. ABA was determined by an indirect enzyme-linked immunosorbent assay (ELISA) test based upon the use of MAC 252 monoclonal antibody, raised against S(+)-ABA (Trivellini *et al.,* 2011). ABA was measured after extraction in 1 mL distilled water overnight at 4ºC (Figure 3, b). Plates were coated with 200 µL per well ABA-4'-BSA conjugate and incubated overnight at 4ºC, then washed three times with 75 mM PBS buffer, pH 7.0, containing 1 g L^{-1} Bovine Serum Albumin (BSA) and 1 mL L^{-1} Tween 20. Then 100 µL of ABA standard solution or sample and, subsequently, 100 µL of MAC 252 (50 µg mL^{-1}) were added to each well and competition was allowed to occur at 37ºC for 30 min. The plates were then washed and 200 µL per well of a secondary antibody [alkaline phosphatase-conjugated rabbit anti-mouse (Sigma-Aldrich, Milano, Italy) in Phosphate Salin Buffer (PSB) at a final dilution of 1:1000] were added (Figure 3,a) and incubated for 1 hour at 37ºC. The plates were washed again and 200 µL per well of *p*-nitrophenyl

phosphate were added and incubated for 30 min at 37ºC. Absorbance at 415 nm was recorded with a microplate reader *(Opsys MR™ Microplate Reader*) along with the software *Windows®Revelation QuickLink™* (Figure 13, c). The samples selected for analysis were collected in four different dates of the growing cicle.

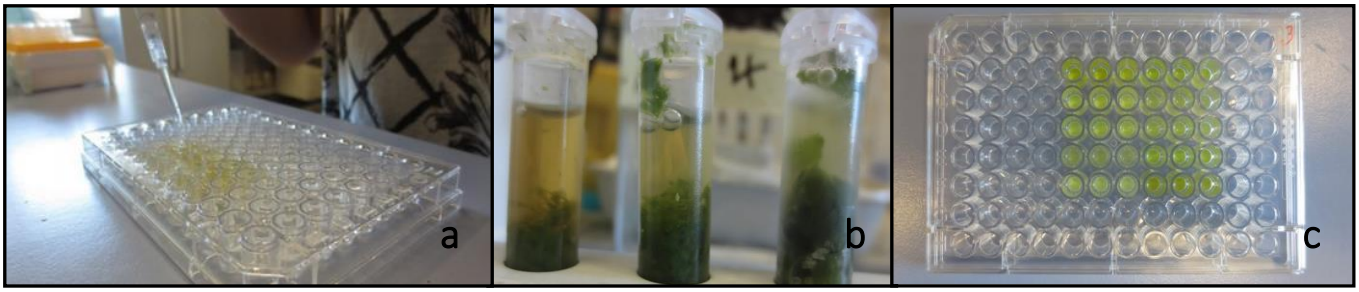

Figure 13 – (a) Adding secondary antibody [alkaline phosphatase-conjugated rabbit anti-mouse (Sigma-Aldrich, Milano, Italy) in Phosphate Salin Buffer (PSB) at a final dilution of 1:1000], (b) Extraction of ABA from *Arundo donax* samples, (c) microtiter plate after adding *p*-nitrophenyl phosphate (reaction complete).

2.6 STATISTICAL ANALYSIS

In this experiment, the 12 rhizotrons were arranged in a completely randomized design, with six replicates for each contrasting water levels [drought at shallow layers (treatment - between 10 and 6% v/v) and field capacity at deep soil layers (control - 23% v/v)]. Two-way analysis of variance (ANOVA) was used for the comparison of all parameters measured between water treatments. ANOVA revealed significant differences among means when $p \leq 0,05$.

3. PRESENTATION OF RESULTS

3.1 GROWTH AND MORPHOLOGY

The relative increase of stem length and diameter were not much affected by drought. Values were identical in both accessions and no differences were found until de end of the experiment. Both water treatments had similar growth and development rates (Figure 14 and 15). Along the experimental period stem length varied between 20 cm and 110 cm and stem diameter varied between 2,5 cm e 6 cm. Until 163 DAT, the plants under drought stress (treatment) presented a slight increase of stem length compared to control plants. Although from 163 DAT onwards stem length of control plants increase. Similarly the same behavior was observed for the stem diameter. In the end of the growing season, around 163 DAT the diameter evolution becomes stable. A two-way ANOVA testing showed no significant differences between treatment and control (Stem length and Stem diameter).

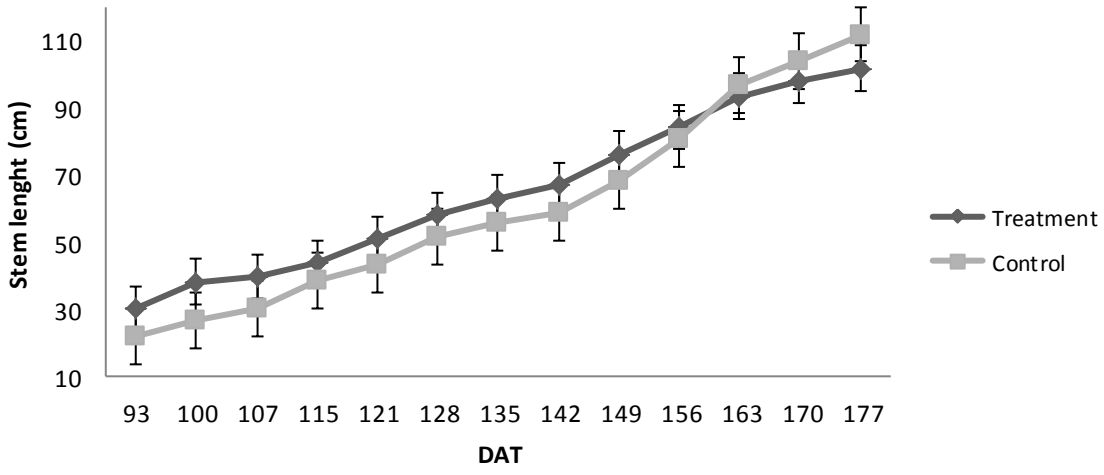

Figure 14 – Effect of drought stress on stem length of *Arundo donax*, continuously grown under well watered control conditions (Control) or subjected to drought stress (Treatment). Values are means.

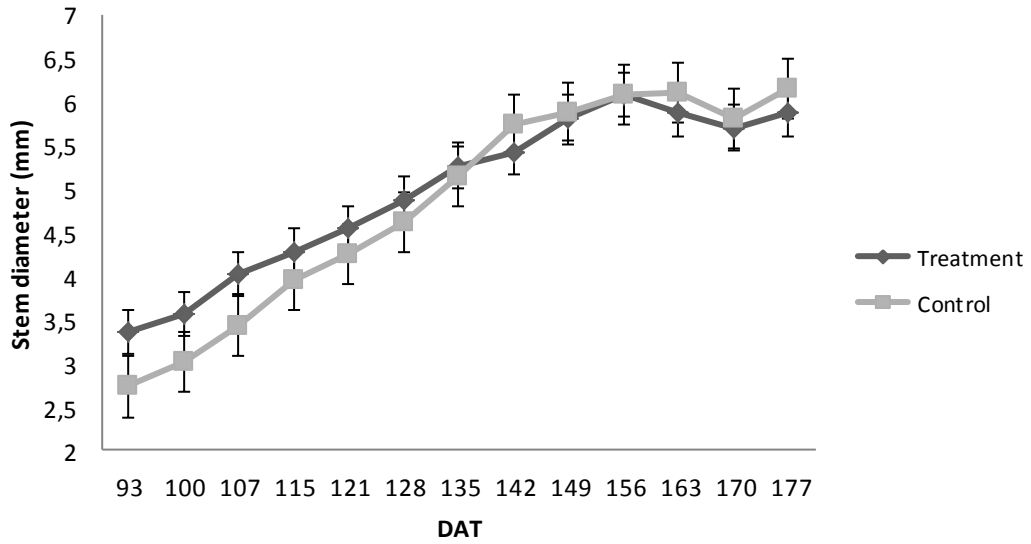

Figure 15 – Effect of drought stress on stem diameter of *Arundo donax,* continuously grown under well watered control conditions (Control) or subjected to drought stress (Treatment). Values are means.

3.2 WATER USE EFFICIENCY AND WATER CONSUMPTION

When drought stress was imposed to topsoil layers (95 DAT) water uptake from deep soil layers increased gradually whereas when both soil layers (top and bottom) were well-watered the main water source remained the topsoil layers (Figure 16) suggesting that *A. donax* has the capacity to adjust the functioning of its deep/shallow root system according to the availability of water. The contribution to the evapotranspiration need from dry topsoil layers was minimal and remained constant from the point when the drought stress was impose onwards. Along the experiment was observed that in the case of the Treatment-top layer (drought) there was a significate de-crease in water consumption when compared with the Control-top layer (well-watered). On the other hand the treatment –bottom layer increase significantly their water uptake compared to the control-bottom layer, as drought becomes more severe.

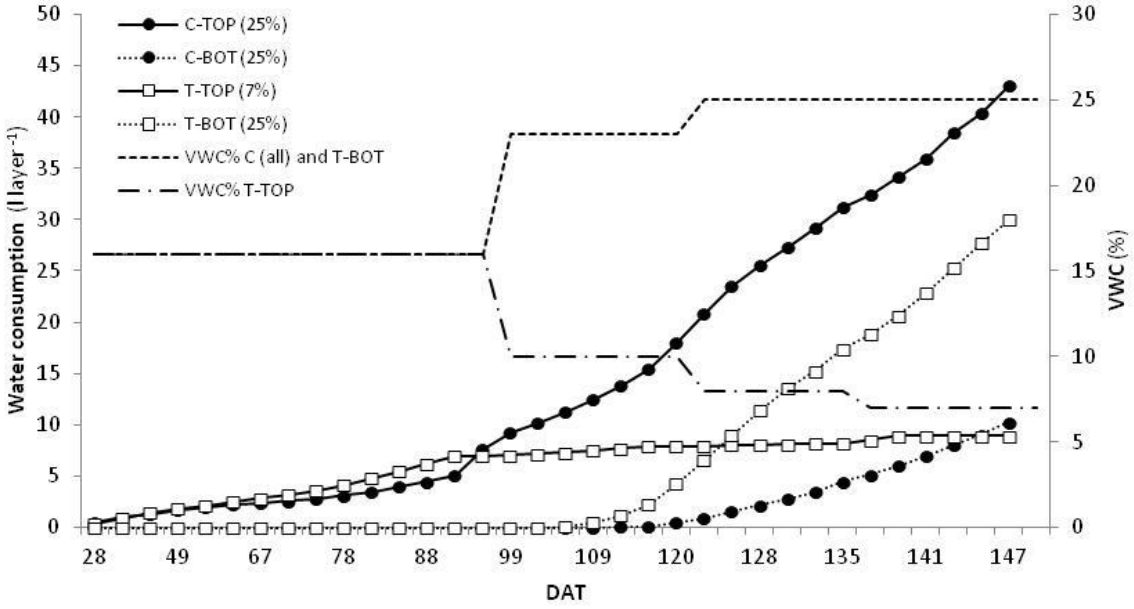

Figure 16 – Average total water used (l top/bottom layer-1) by *A. donax* subjected to contrasting water level at top and bottom layers. Control upper (C-TOP) and bottom (C-BOT) sections, Treatment upper (T-TOP) and bottom (T-BOT) sections. Dashed lines represents respectively the Volumetric Water Content imposed to drought section of Treatment (– · –) and control and treatment bottom layer (–––). DAT means Days After Transplant.

3.3 LEAF LEVEL GAS EXCHANGE

The physiological activity of both crops under well-watered and drought conditions are presented in Figure 17 and 18. Values and variation of net photosynthesis (Figure 18) and stomatal conductance (Figure 17) along the experiment were similar in accessions, control and treatment. Pn values under treatment were similar to those observed for control plants. No statistically significant ($p \geq 0,05$) were observed in both cases. The stomatal conductance (*gs*) varied between 250 $mmolm^2s^{-1}$ and 150 $mmolm^2s^{-1}$. Moreover, the *gs* at 150 DAT was reduced for both crops over time. Following a similar pattern to the photosynthetic rate, that decreased also over time. How-However the photosynthetic rate for drought-treatment crop was lower than control-crop, this is due to the reducing of CO_2 assimilation and transpiration in drought treatments. Stomatal limitation was one of the factors causing photosynthetic rate decrease in treatment-plants (drought stress plants).

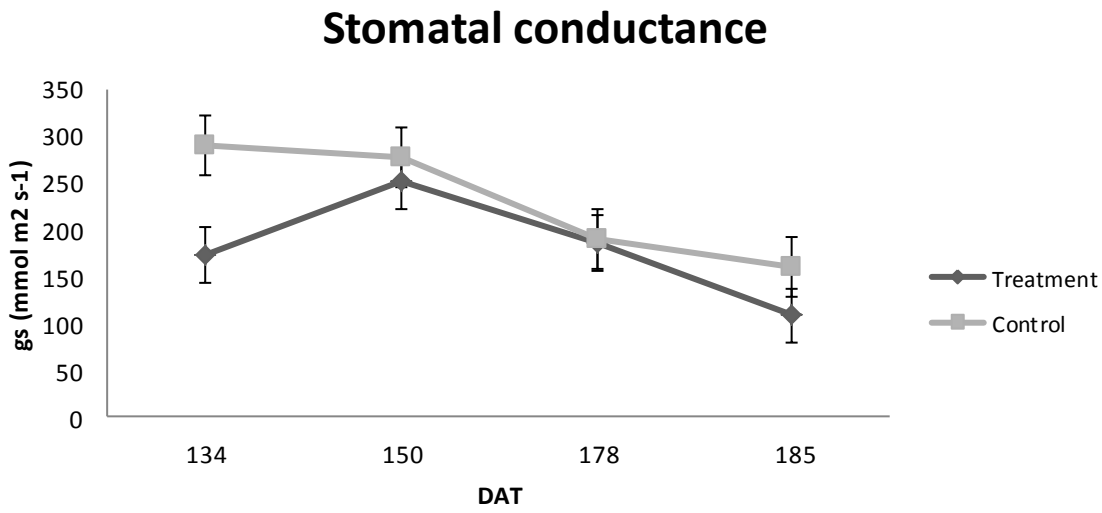

Figure 17 – Effect of drought stress on stomatal conductance (g_s) of *Arundo donax*, continuously grown under well watered control conditions (Control) or subjected to drought stress (Treatment), from 134 DAT to 185 DAT. Values are means.

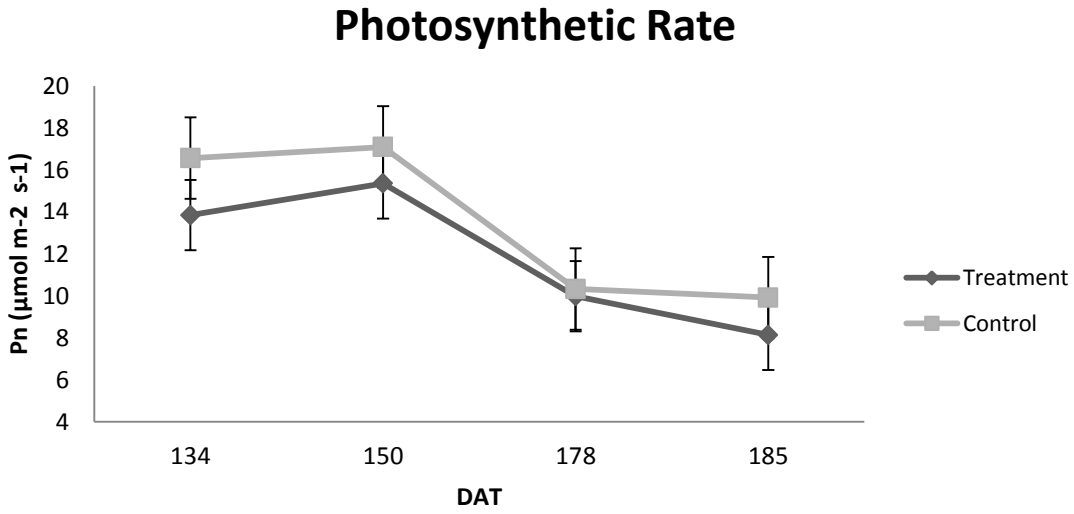

Figure 18 – Effect of drought stress on photosynthesis (P_n) of *Arundo donax*, continuously grown under well watered control conditions (Control) or subjected to drought stress (Treatment), from 134 DAT to 185 DAT. Values are means.

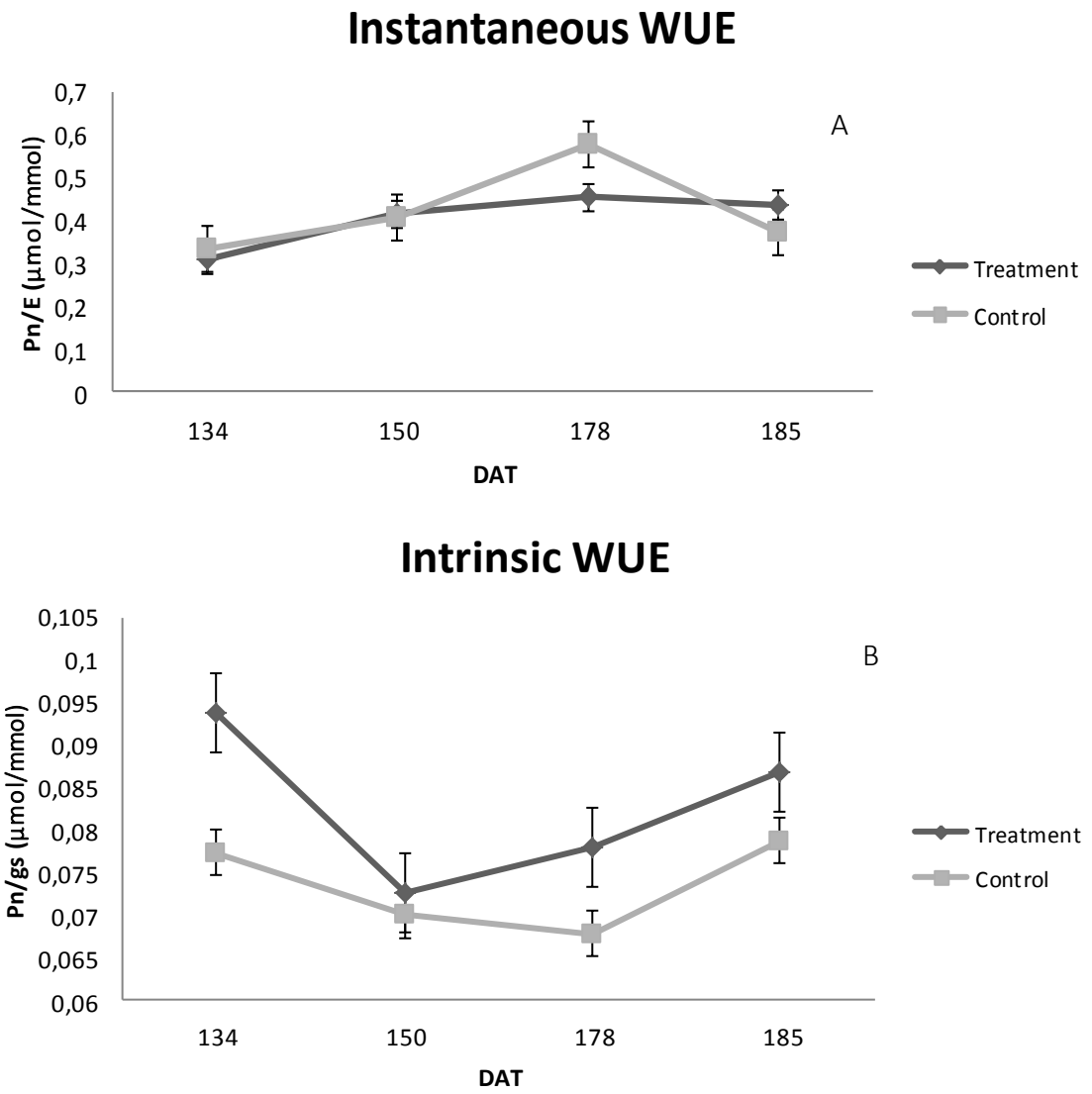

Figure 19 – Effect of drought stress on (A) instantaneous water use efficiency (P_n/E) and (B) intrinsic water use efficiency (Pn/gs) of *Arundo donax*, continuously grown under well watered control conditions (Control) or subjected to drought stress (Treatment), from 134 DAT to 185 DAT . Values are means.

Instantaneous WUE (Pn/E) is leaf-level carbon gained compared to water loss and intrinsic WUE (Pn/gs) is used to compare photosynthetic properties independent of evaporative demand. Plots of these WUE measurements illustrate similarities in the case of instantaneous WUE (Figure 19, A), where both crops, control and treatments, showed similar performances (no significant differences were showed). However for instantaneous WUE (Figure 19, B) the treatment crop had higher values than the control crop. In this case significant differences between control and treatment plants were confirmed. Measurements observed for both crops shown that the Photosynthetic rate (assimilation) was reduced for treatment-plants compared to control plants (Figure 18) but water use efficiency remained comparable to controls, in the case of instantaneous WUE (Figure 19, A) and superior to controls for intrinsic WUE , indicating control of stomatal closure over water losses by transpiration. (Figure 19, B).

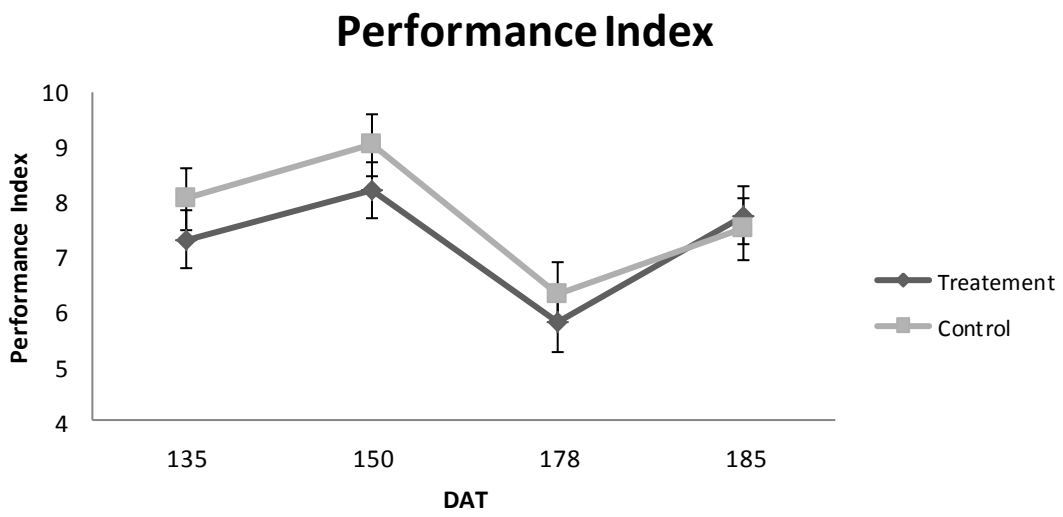

Figure 20 – Effect of drought stress on Performance Index based on leaf fluorescence measurements of *Arundo donax*, continuously grown under well watered control conditions (Control) or subjected to drought stress (Treatment), from 134 DAT to 185 DAT. Values are means. *Performance index is an integrative parameter that measures the efficiency of the photosystem II, including the density of fully active reaction centers, the efficiency of electron movement and transport, and the conversion of excitation energy into electron flow.

The performance index, a plant vitality index derived from leaf fluorescence measurements, confirmed the higher capacity of drought Treatment crop to sustain its physiological activity as it would do it under optimal growing conditions. Even though the absolute performance index values were higher in control plants (Figure 20), no statistically significant differences were found between both crops. The transpiration rate (Figure 21) was lower for drought treatment compared to control. However no significant differences were showed between control and treatments crops. The higher value of transpiration was on 150 DAT. From this date forward transpiration rate experience a gradual decrease for both control and treatment crops.

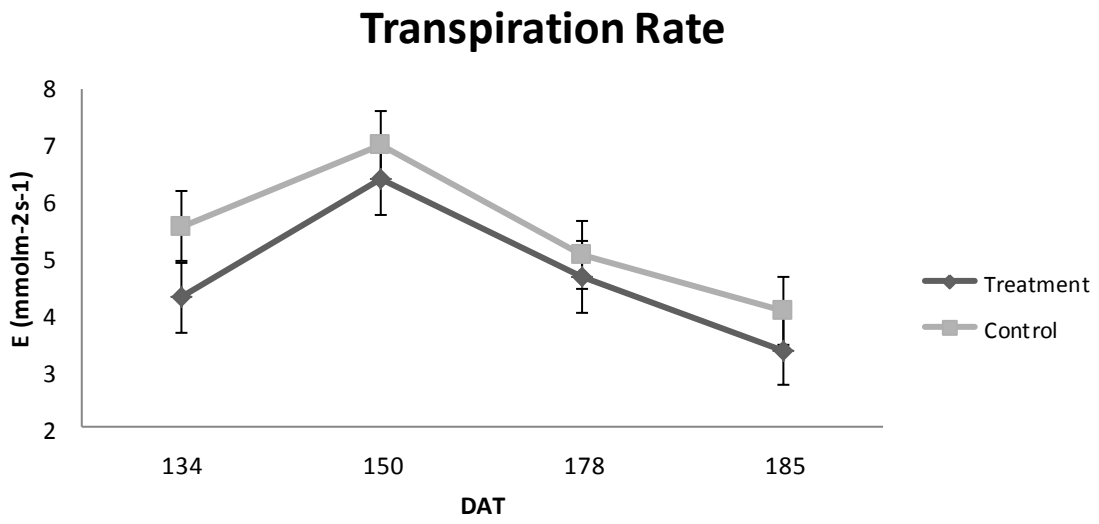

Figure 21 – Effect of drought stress on transpiration rate of *Arundo donax*, continuously grown under well watered control conditions (Control) or subjected to drought stress (Treatment), from 135 DAT to 185 DAT. Values are means.

The photochemistry of photosynthesis, as monitored by PSII efficiency estimated by chlorophyll fluorescence, showed similar performances between treatment and control plants. The maximal quantum yield of PSII (Fv/Fm), Figure 22, was not significantly affected under drought stress (treatment crop). From 150 DAT until 178 DAT a slight decline in Fv/Fm plot was observed in both treatment and control plants. There was no significant difference between both crops over the duration of the experiment. No decline was observed in plants-treatment performance as the soil and plant moisture depleted and drought became increasingly severe compared to control. No significant differences were showed between both accessions. As for the relative water content (RWC) an increase in RWC for the treatment plants was observed (Figure 23). For the control plants RWC was stable in the two dates that data was collected.

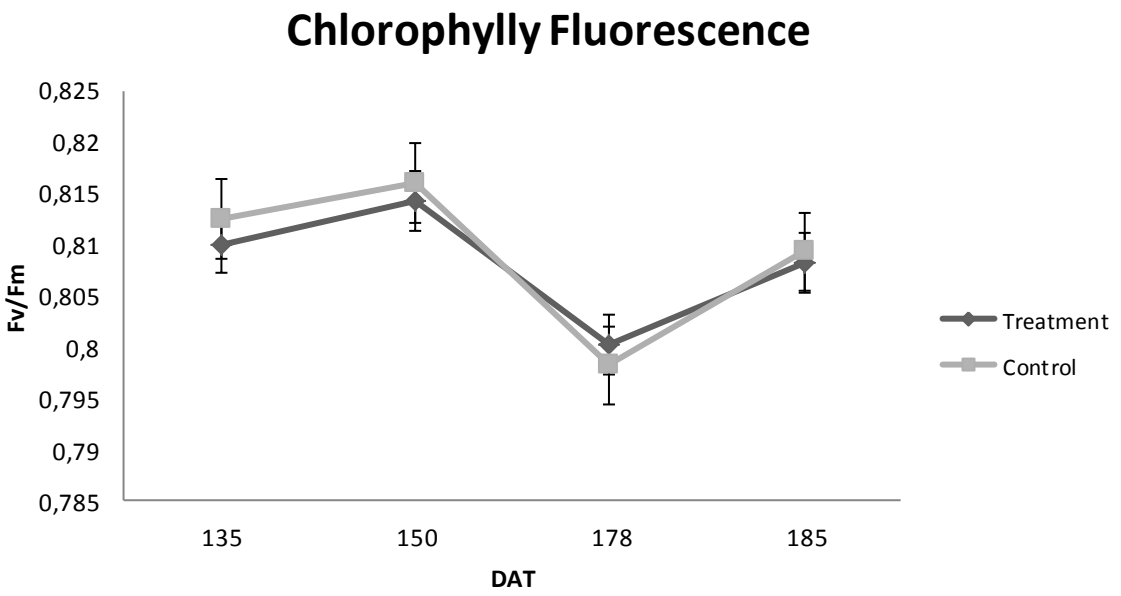

Figure 22 – Effect of drought stress on chlorophyll fluorescence (Fv/Fm) of *Arundo donax*, continuously grown under well watered control conditions (Control) or subjected to drought stress (Treatment), from 135 DAT to 185 DAT. Values are means.

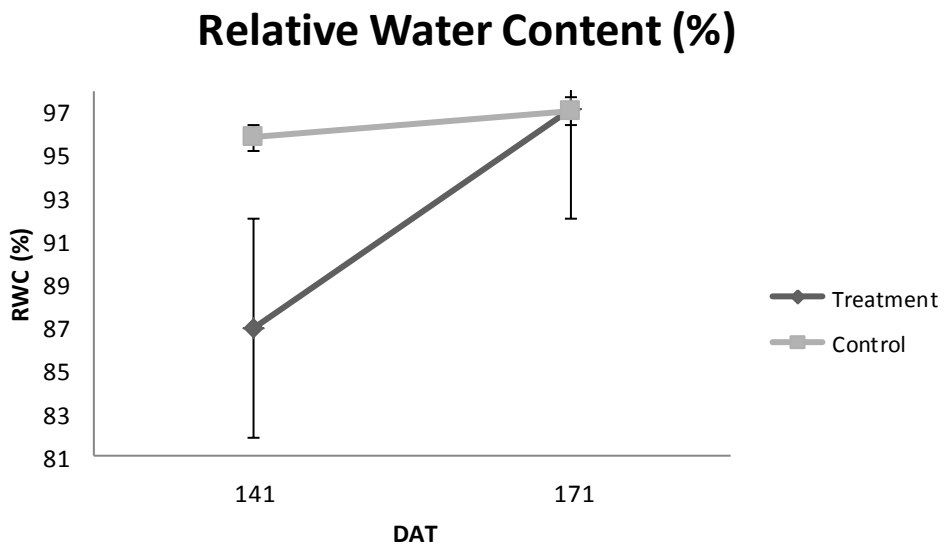

Figure 23 – Effect of drought stress on relative water content (RWC) of *Arundo donax*, continuously grown under well watered control conditions (Control) or subjected to drought stress (Treatment), corresponding to 141 AND 171 DAT. Values are means.

3.4 ROOT WATER UPTAKE EFFICIENCY

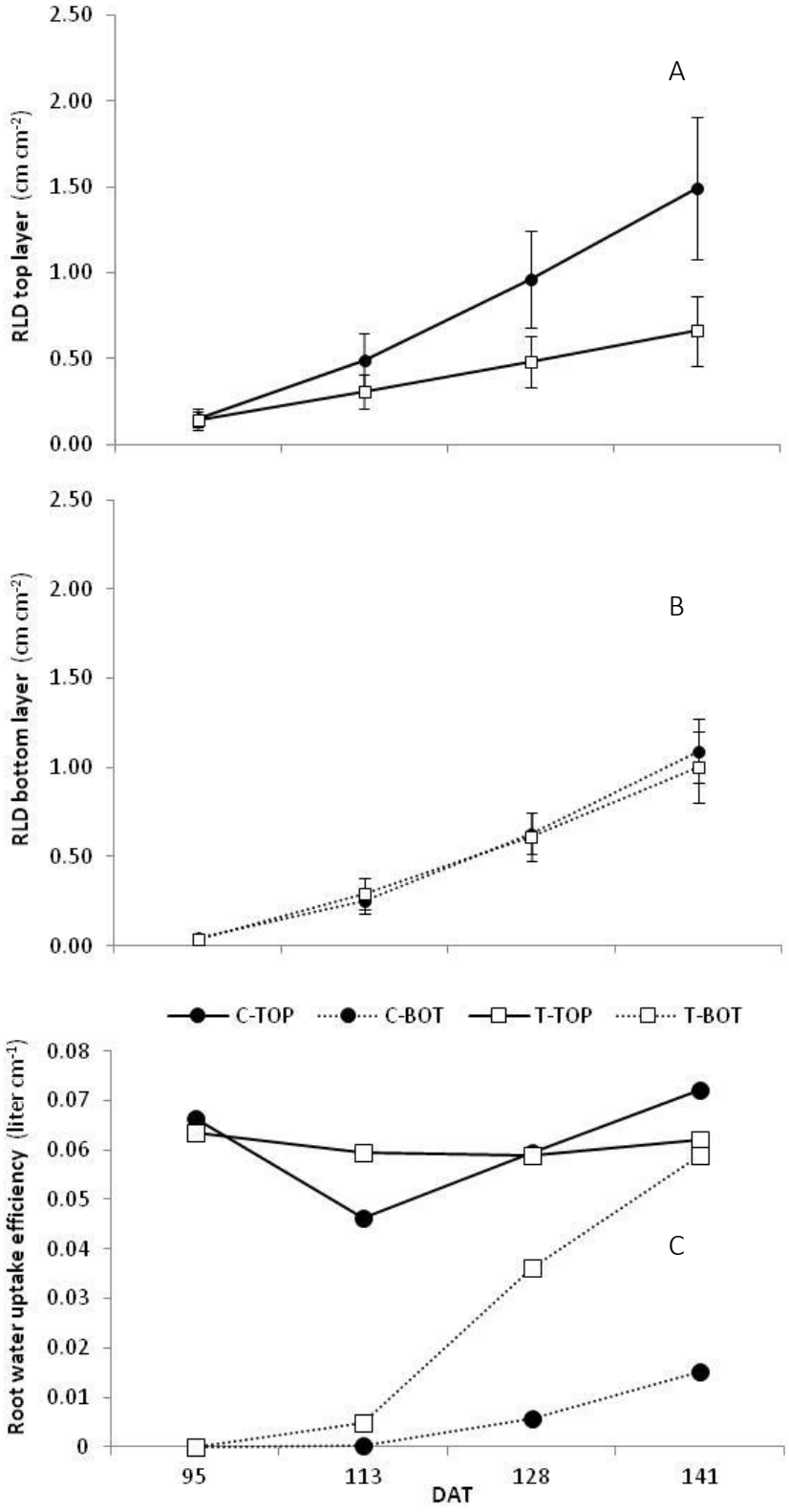

Figure 24 – Root length density (RLD) and root water uptake efficiency of *A. donax* subjected to contrasting water level at top and bottom layers, Control (C) and Treatment (T). Values are means.

The effects of drought in the topsoil layers on the deep water uptake characteristics of the two accessions are presented in Figure 24. In general, a gradual increasing difference in root length density (RLD) was observed between control and treated topsoil layers as the stress treatment advanced. On the other hand in the bottom layers RLD values were always the same in both treatments (Figure 24, B). However, despite the significant increase in water uptake from deep layers of plants subjected to drought in upper layers (Figure 24, C) its RLD was not increased suggesting and increased efficiency in root water uptake. In fact root water efficiency was significantly increased from DAT 113 onwards. Root water uptake from bottom treatment layer was higher compared to top control layer. From 113 DAT, T-Bottom layer was able to continue uninterruptedly its increasing relative water uptake pattern, while C-Bottom layer showed almost constant values with time. However, C-Top layer and T-Top layer showed a similar evolution, both were almost even over time.

3.5 QUANTIFICATION OF ABSCISIC ACID

The quantification of ABA represented a challenge in this experimental assay because there was no protocol of quantification of abscisic acid in *Arundo donax.* It was observed that both treatment and control plants has similar plots for the concentration of Abscisic acid. Drought stress induced biosynthesis of leaf ABA in treatment plants but no correlation can be made between drought and increase of ABA concentration in leaves, because control plants had similar concentrations of ABA over time suggest that there are no differences between treatment and control plants in terms of ABA physiology. For both cases the maximum value was reach on 171 DAT and then decreased (Figure 25). For the verification of this new method were measured two lots of samples of maize that was already known that one lot had higher signal of ABA and the second lot of maize leaves had lower signal of ABA. Another method of analysis, HPLC or GS, should be used to analyze the same samples in order to validate the method of quantification used for the achievement of these results.

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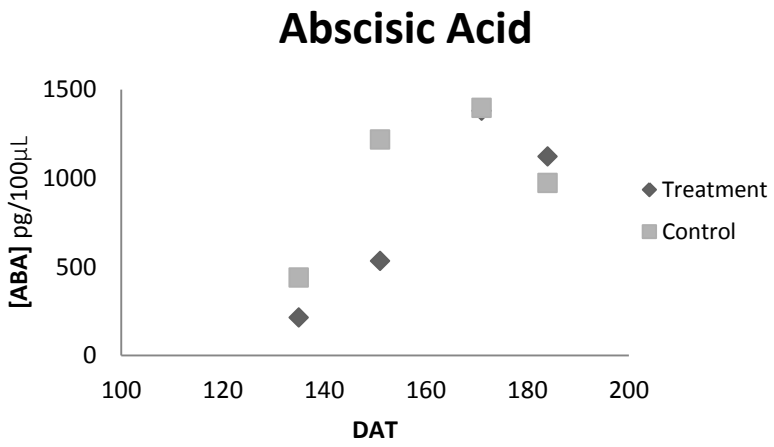

Figure 25 – Effect of drought stress on abscisic acid concentration in *Arundo donax* leaves, continuously grown under well watered control conditions (Control) or subjected to drought stress (Treatment). Values are means.

4. DISCUSSION

In this experimental assay was characterized *Arundo donax* physiology and photosynthetic response to water stress imposed in top soil layers. It was found no, or only minor, effect of the drought top soil layers on the morpho-physiological response of *Arundo donax* compared to control plants. The maintenance of *Pn* values under drought stress from top soil layers result in an increase of intrinsic water use efficiency of the whole plant and increase also root water uptake of bottom deeps roots. This water conservation strategy can justify the maintenance of high RWC even under severe drought stress. It is also possible that the osmotic adjustment capacity has contributed to maintain a high water content of tissues (Silva *et al.,* 2010).

Photosynthesis is the primary process to be affected by drought (Chaves e Oliveira, 2004). In this study no grown (length and diameter stem) reduction was observed under moderate or severe drought stress imposed to top soil layers. This suggests that even at reduced soil water availability, *Arundo donax* plants are able to grow. Moreover, results showed that *Arundo donax* is able to change its water sources depending on its availability and therefore supply to the canopy all of its evapotranspiration needs to maintain unvaried its growth, development and physiological functions. These results agree with previous findings, that plants on drought treatments were able to continuously grow, although at a slower rate than fully irrigated plants (Achten *et al.,* 2010).

A reducing of stomatal conductance was not related to the relative content of water since RWC on drought top layer increase, suggesting that stomata are responding to other factors to prevent water loss transpiration. Moreover, there is strong evidence that *gs* responds to soil water content rather than leaf water turgor/content (Jones *et al.,* 1992). In line with previous findings, for *Arundo donax*, this study suggests that two main strategies to endure drought stress are via stomatal control and limiting transpiration (Silva *et al.,* 2010). Although stomatal closure is a fast mechanism that reduces water loss in *Arundo donax*, increasing the resistance of CO_2 diffusion and carboxylation efficiency is only affected after a reduction of stomatal conductance of approx. 50%. This shows the resilience of the photosynthetic apparatus to drought and points CO_2 limitation being the main factor responsible for the decreased of *Pn* from 150 DAT. These findings also suggests that *Arundo donax* can be grown under water deficit in the top soil layers maintaining high photosynthetic capacity with reduced water loss, due to the increased of intrinsic WUE. The high water use efficiency is a direct consequence of the decrease in *gs,* which is a typical response observed in other species when subject to mild drought stress. In our case study a synchronized reduction of stomatal conductance and net assimilation (*Pn*) were observed since 150 DAT, which was reported in Silva *et al*. (2010).

As water stress inhibits photosynthesis and stomatal conductance, the ability of chlorophyllous cells to utilize the absorbed light and dissipate excessive energy is reduced, because the electron transport in the photosystem II (PSII) is inhibited and/or the reaction center is oxidatively damaged (Rao *et al.,* 2006). The inhibition on the PSII electron transport has been correlated with a loss of chlorophyll a fluorescence yield. In the present study, the photochemistry efficiency, expressed as chlorophyll a fluorescence performance index, was similar for crops, treatment and control.

The performance index is an index of photosynthetic performance on the basis of light absorption on small portion of the leaf. It does not necessarily reflect the performance of the whole plant as this is represented by a summation of the effects of the plant interactions over growing period. However, the same patterns found between performance index, gas exchange and plant growth suggest that whole *Arundo donax* performance was no affected by the drought treatment. PSII efficiency follows this same pattern and also decreases its value since 150 DAT as *gs* and *Pn*. At severe drought, 185 DAT, the deep soil layers can maintain the same pattern of well-water root.

Previous research revealed that mature *Arundo donax* plants are very drought tolerant (Mann *et al.,* 2012). Limiting transpiration appears to be the primary mechanism for delaying water-stress. Similarly, was found that for other C3 crops stomatal closure and reduced transpiration was the primary mechanisms for drought avoidance (Liu *et al.*, 2004). Since 150 DAT were observed a decreased in transpiration rate that also offers a possible explanation for the slight decrease in Fv/Fm since 150 DAT. Reduced transpiration means that evaporative cooling of the leaf surface is diminished. As drought progressed, transpiration was further restricted by water-stress. Decreasing transpiration is a stress protection mechanism that limits water loss and lessens damage associated with desiccation.

Aboveground biomass in both crops followed a similar pattern to the developmental progress suggesting that deep roots can alone reach same values of canopy development that well-watered root. It is well known that aboveground biomass development has a critical effect on root development, and the equilibrium between the two phases could change in time, water availability, and other factors. In the present study although root water uptake efficiency of bottom layers that had the drought-top layer was increased continuously compared to bottom-control, there no evidence based in RLD bottom layer that suggest that *Arundo donax* has the capacity in situations of water stress to develop a larger and more evenly distributed root system, (Zegada-Lizarazu *et al.,* 2012) in maize and sorghum plants. But can be observed that *Arundo donax* increase root water uptake efficiency, in stress conditions, that allows the plant to maintain all the physiological and photosynthetic rates as the same as a well-watered plant. The

increased relative water uptake capacity may have helped *Arundo donax* to improve its leaf water status, transpiration rates, and plant viability. On the other hand, the increase on root water uptake was sufficient to maintain its physiological activity, suggesting that the drought adaptation strategy of increasing the root water uptake efficiency employed by *Arundo donax* is as effective as increasing the total root length density as it was reported in studies with sweet sorghum (Zegada-Lizarazu *et al.,* 2012). Therefore the sustained physiological activity and lower detrimental effects on aboveground biomass production in *Arundo donax* could be related to its improved WUE but not to increased RLD.

From literature it is known that water passively move into the roots of a plant, through the apoplastic and cell-to-cell pathways, in response to water potential gradients, but under drought stress the predominance of one or the other is enhanced depending on the species and environmental conditions. In general, at lower soil water potentials water movement towards the roots is mainly driven by active pumping of solutes (photosynthates) in the root, which creates an osmotic gradient in the cell-to-cell pathway (Maurel *et al.,* 2002). In *Arundo donax* it was indicated that the predominance of either path could be enhanced depending on the prevailing driving forces. Moreover, it has been demonstrated that apoplastic water movement cannot maintain osmotic driving forces and that drought stressed maize roots exhibit pronounced levels of suberization (Maurel *et al.* 2002) that in turn reduce significantly the apoplastic water absorption capacity of a plant (Steudle *et al.,* 2000). Then in the present case, drought stress probably enhanced the active water uptake pathways in *Arundo donax* even though its lower capacity to adjust osmotically, compared to sweet sorghum (Zegada-Lizarazu *et al.,* 2012) in which both the apoplastic and cell-to-cell pathways may work well in sweet sorghum through its more extended root system. It is suggesting that a large proportion of photosynthates accumulating in *Arundo donax* roots were used to adjust osmotically and enhance water uptake on a restricted root volume, while in the case of sweet sorghum, the photosynthates were invested to enlarge its root system, thus reduce the resistance to water fluxes. In past studies drought resistance in sweet sorghum is associated with its capacity to improve its WUE, enhanced root length density, and with the ability to maintain high leaf water potential and physiological activity under drought stress (Maurel *et al.,* 2002).

It was been expected that photosynthesis and stomatal conductance were inversely associated with ABA content, in heavily stressed leaves for drought treatment plants. However this pattern was not observed, instead a similar pattern between treatment and control was observed indicate that ABA doesn't play a very important role in drought of *Arundo donax.*

5. CONCLUSION AND FUTURE WORK

Second-generation bioenergy's attempt to achieve carbon-neutrality and avoid direct competition in commodities markets by focusing on novel crop species like *Arundo donax* that have high production rates with minimal nutrient inputs. However, novel crop species will compete directly or indirectly for natural resources including arable land and freshwater. *Arundo donax* is an example of a novel feedstock whose impacts on water-resource were far unknown. The results of this experiment provide some date for estimating whole plant WUE, as well as strong indications of physiological consequences. The gas-exchange data suggest that *Arundo donax* has relatively high transpiration rates and will therefore use more water than many C3, as well as C4 biomass feedstocks. The two accessions of *Arundo donax,* treatments and control, were compared and showed no marked morpho-physiological differences in response of drought stress in TOP soil layers, forcing deep roots to compensate the water deficit in Top layers. Both photosynthetic and hormonal rates were maintained during water deficit. Furthermore, this species has shown to improve water use efficiency along drought periods. This results support the idea that *Arundo donax* is appropriate for cultivation in areas with limited water availability.

Is clearly more work has to be done to understand how *Arundo donax* will respond to climate change, and how these results will play out in situ, the findings of this study are significant because represents one of the few studies regarding *Arundo donax* WUE and stress tolerance through time with deep root water uptake ability. Therefore, this study aims to contribute to our greater understanding of the impact of *Arundo donax* cultivation as a biomass feedstock, and to inform sustainable management regarding the impacts of this species on water resources.

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